Modulation of Oxygen-Regulated Erythropoietin Expression

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Table of Contents

Summary

Erythropoietin (Epo) is the key hormone regulating red blood cells homeostasis and is produced in the foetal liver and adult kidney. Epo plasma levels physiologically increase in response to hypoxia and anaemia, following the activation of the HIF (hypoxia-inducible factor) pathway. In pathological conditions, such as chronic kidney disease (CKD) or erythrocytosis, Epo levels markedly decrease or increase, respectively, leading to a variety of complications. Despite extensive efforts to isolate the peritubular interstitial renal cells responsible for oxygen-sensing and Epo production, no kidney-derived cellular model capable of expressing Epo in a hypoxia-inducible manner is available so far. Thus, only the well-established human hepatoma cell lines HepG2 and Hep3B and some neuronal cell lines (such as Kelly and SH-SY5Y) expressing oxygen-dependent Epo can be used to investigate Epo gene regulation *in vitro*.

The lack of an appropriate renal cell model explains the poor understanding of the *cis*-acting element regulating kidney-specific Epo expression (kidney-inducible element or KIE), as defined by transgenic animal experiments. By comprehensive *in silico* analysis we identified a novel distal hypoxia response element (HRE) located upstream of the *EPO* gene, within the KIE, and named it Epo 5'-HRE, in contrast to the well-known 3'-HRE, located downstream of the gene and responsible for liver-specific Epo induction. Functional studies showed that the 5'-HRE is mainly responsive to HIF-2α isoform. We additionally implemented the innovative CRISPR/Cas technology to mutate the endogenous 5'- and 3'-HREs in Hep3B cells in order to evaluate the contributions of each element in regulating oxygen-dependent Epo expression. Furthermore, we screened a number of candidate factors, either known or novel regulators of the HIF pathway, for a potential effect on Epo expression using RNA interference-mediated or overexpression approaches in combination with newly developed Epo-reporter assays to study the 5'-HRE.

In erythrocytosis, a disease characterized by inappropriately high Epo plasma levels, Epo synthesis appears dysregulated. Whereas in the majority of congenital secondary erythrocytosis patients the cause is unknown, a number of mutations in genes involved in the oxygen-sensing pathway, such as HIFs and prolyl-4-hydroxylase domain proteins (PHDs) have been identified. As part of a collaboration with the group of Dr. Betty Gardie (Nantes, France) we functionally characterized several novel mutations in the *PHD2* gene causing familial erythrocytosis and could group these mutations into two main categories in terms of their effect on HIF regulation. Notably, some of the mutations did not lead to impaired PHD2 activity, at least in our *in vitro* assays.

An additional layer of modulation of Epo production is represented by humoral factors, including peptides and hormones. We tested selected peptides in Hep3B for a potential role in Epo regulation and complemented this approach by performing a large-scale screening of a peptide library derived from human haemofiltrates using different HIF-reporter cell lines. Results from the first, complete screening are promising and showed the presence of both “inhibitory” and “activatory” fractions in terms of HIF functionality.

In conclusion, this PhD work focused on the hypoxic regulation of the *EPO* gene on multiple levels and described for the first time a novel HRE, possibly representing the hypoxia-inducible element responsible for kidney-specific Epo induction. The newly identified Epo 5’-HRE represents the basis for subsequent studies to investigate the effect of a number of factors and peptides on oxygen-dependent Epo regulation.

Zusammenfassung

Erythropoetin ist das Schlüsselhormon, das die Homeostasis der roten Blutkörperchen reguliert und wird in der fetalen Leber und der adulten Niere produziert. Die Epokonzentration im Plasma erhöht sich physiologisch in Antwort auf Hypoxie und Anaemie im Anschluss an die Aktivierung des Hypoxieinduzierter Faktor (HIF) Signalweg. Unter pathologischen Bedingungen, wie chronisches Nierenversagen oder Erythrozytose sinkt oder steigt die Konzentration von Epo messbar, was zu verschiedenen Komplikationen führt. Trotz Anstrengungen, die peritubulären interstitialen Zellen, die für die Sauerstoffwahrnemung und die Epoproduktion verantwortlich sind, zu isolieren, ist bisher kein Zellmodel aus der Niere verfügbar, das fähig ist Epo in einer hypoxieabhängigen Art und Weise zu exprimieren. Deshalb können nur die gut etablierten Hepatoma Zelllinien HepG2 und Hep3B und einige neuronale Zelllinien wie Kelly und SH-SY5Y, die Epo in Abhängigkeit von Hypoxie exprimieren, für die Erforschung der Epogenregulation verwendet werden.

Das Fehlen eines passenden Nierenzellmodells erklärt das geringe Verständnis für das cis agierende Element, welches die nierenspezifische Epoexpression reguliert (das Niereninduziertes Element, KIE), wie es durch Experimente mit transgenen Tieren definiert wurde. Durch eine umfassende Analyse *in silico* haben wir ein neuartiges distales Element für die Hypoxieantwort untersucht, welches upstream des Epogens innerhalb des KIE liegt, und haben es Epo 5'-HRE gennant. Im Unterschiedt zu dem wohl bekannten 3' -HRE, welches downstream des Gens liegt und sich für die leberspezifische Epoinduktion verantwortlich zeigt. Funktionelle Studien haben gezeigt dass das 5'-HRE hauptsächlich auf die HIF-2α Isoform reagiert. Wir implementierten zusätzlich die innovative CRISPR/Cas Technologie, um die endogenen 5'- und 3'-HREs in Hep3B zu mutieren und so den jeweiligen Beitrag von den beiden Elementen zur Sauerstoff abhängigen Epoexpression zu evaluieren. Ausserdem untersuchten wir einige neue und bekannte Faktoren in der HIF-Singnalwegregulation auf einen potentiellen Einfluss auf die Epoexpression in dem wir ein auf RNA-Interferenz und Überexpression basierendes Epo-Reporterassay verwendeten.

In Erythrozytose, eine Krankheit, die durch einen abnorm hohen Epoplasmastand charkterisiert ist, scheint die Synthese von Epo dereguliert. Obwohl bei der Mehrheit der Patienten mit angeborener sekundärer Erythrozytose der Ursprunge unbekannt ist, wurden einige Mutationen in Genen, wie den HIFs und Prolyl-4-hydoxylase Domänen Proteinen (PHDs), gefunden, die in den Sauerstoffwahrnehmungssignalweg involviert sind. Als Teil einer Kollaboration mit der Gruppe von Dr. Betty Gardie (Nantes, Frankreich) haben wir die Funktion von mehreren neuen

Mutationen im PHD2 Gen charkterisiert, die familiäre Erythrozytose verursachen, und haben diese Mutationen im Bezug auf ihren Effekt auf HIF in zwei Hauptklassen eingeteilt. Bermerkenswerterweise führten einige dieser Mutationen zumindest in unserern Versuchen nicht zu einer Störung der PHD2 Aktivität.

Eine zusätzliche Ebene der Regulation der Epoproduktion wird representiert durch humorale Faktoren, wie Peptide und Hormone. Wir haben eine Auswahl an Peptiden auf eine mögliche Rolle in der Eporegulation in Hep3B getestet und diesen Ansatz mit einer breit angelegten Untersuchung einer Sammlung von Peptiden aus menschlichen Hämofiltraten ergänzt, indem wir verschiedene HIF-Reporter Zelllinien verwendeten. Die Resultate aus einer ersten Reihe dieser Untersuchungen sind vielversprechend und zeigen die Anwesenheit von im Bezug auf die Regulation von HIF sowohl inhibierenden als auch aktivierenden Fraktionen.

Zusammengenommen konzentrierte sich diese Doktorarbeit auf die hypoxische Regulation von Epo auf verschiedenen Ebenen und beschreibt zum ersten Mal ein neues HRE, welches möglciherweise das Element representiert, welches für die Nierenspezifische Epoinduktion verantwortlich ist. Das neu identifizierte Epo 5'-HRE legt die Basis für weitere Studien mit dem Ziel den Effekt einer Vielzahl von Faktoren und Peptiden auf die Sauerstoff-abhängige Eporegulation zu untersuchen.

1. Introduction

Erythropoietin (Epo) is often a topic of interest for the world's media because of its wide use as a doping agent in a variety of sport disciplines, especially when endurance is involved. Why is there such a big illegal market for recombinant Epo? What are the benefits and the side effects of such treatment? Recombinant human Epo (rhEpo) was first approved by the American Food and Drug Administration in 1989 as a therapeutic approach to treat patients with anaemia, i.e. when the number of red blood cells (RBCs) or the concentration of haemoglobin (Hb) is not sufficient to support appropriate oxygen (O_2) delivery to tissues. Increasing the haematocrit (Hct, volume of RBCs on the total blood volume) to a certain extent increases the oxygen-carrying capacity of the blood and has the proven effect of enhancing the athlete's performance too, at least for a certain range of Hct values. The number of athletes that have been tested and found positive for doping increased exponentially after the introduction of rhEpo on the market in the early 1990's and nowadays new doping routes, including alternative erythropoiesis-stimulating agents and gene transfer approaches, are becoming increasingly popular and difficult to detect for the regulatory agencies. One of the most famous cases of doping concerns cycling: during the Tour de France in 1999 for example most of the athletes on the American national team, including famous Lance Armstrong, were found to be positive to performance-enhancing substances, mainly Epo. Less known is probably the case of "natural doping" of the Finnish cross-country skier Eero Mäntyranta, who suffered from a genetic form of polycythemia, or eythrocytosis, a clinical condition leading to excessive number of RBCs. The oxygen-carrying capacity of his blood was increased of up to 50%, a large advantage that led him to win several world and Olympic medals in the 60's and 70's. On the other hand, erythrocytosis can have deleterious effects due to the increased viscosity of the blood and subsequent increased risk of blood vessel occlusion, or thrombosis, and is definitely considered a haematological disease. Therefore, it seems important to maintain Epo levels within a specific range but what is the physiological meaning of a factor regulating the number of RBCs? RBCs, or erythrocytes, are enucleated cells of the blood containing the O_2-binding protein haemoglobin and represent the mechanism used by higher vertebrates to deliver oxygen to every single cells of the body. The amount of oxygen dissolved in the plasma would in fact not be sufficient to meet the metabolic demand of the entire organism. O_2 is used by the mitochondrial respiration machinery of the cell to produce energy in the form of adenosine triphosphate (ATP) molecules and conditions of reduced oxygen tension (or hypoxia) are extremely dangerous for life. Hypoxic conditions are not so far from every-day life as one would expect: going to high altitude (>2500 m on sea level) is already a challenge for our body and it is interesting to know how whole populations, such as Tibetans and Peruvians, have adapted in order to live in conditions of chronic

hypoxia. Moreover, hypoxia is a hallmark of several diseases such as chronic kidney disease and cancer: accumulating evidences in literature suggest that the hypoxic microenvironment of tumours for instance supports the growth of the neoplastic tissue, as well as increases angiogenesis and chemoresistance. Organisms have developed mechanisms to sense changes in partial pressure of oxygen (pO_2), to trigger activation of the hypoxia-inducible factor (HIF) pathway at the cellular level and consequently to produce changes at the systemic level in order to guarantee adequate O_2 delivery to peripheral tissues. Such adaptive mechanisms include Epo production which will in turn increase the Hct and allow the blood to uptake as much oxygen as possible from the environment. Basal production of Epo is also required for the normal turn-over of RBCs. Epo is thus an essential hormone for life and understanding its regulation is an important challenge, not only to fight against illegal doping in sports but mainly to provide improved treatments for patients with inappropriate Epo synthesis.

1.1 Physiology of Epo production

Epo is the most important regulator of RBCs homeostasis. In adults, the amount of circulating RBCs is fairly constant due to a balance between daily produced and removed RBCs. The bone marrow is responsible for erythrocyte production (erythropoiesis), whereas the mononuclear phagocyte system of the spleen, liver and lymph nodes removes the ~120-days old cells from the blood stream (Wenger and Kurtz, 2011). In condition of reduced oxygenation (hypoxia) or Hb content (anaemia), the oxygen-carrying capacity of the blood is concomitantly reduced and RBC production by the bone marrow can be increased up to 8-fold within a few days. This effect was described already in the 19th century by Francois-Gilbert Viault, who observed increased RBC count in people travelling at the altitude of 4200 m in the highlands of Peru (Viault, 1890). In 1905 Paul Carnot and his assistant Clotilde Deflandre proposed for the first time the possibility of a hormonal regulation of erythrocyte production and in the middle of the 20th century evidences from anaemic animals confirmed the existence of a humoral factor responsible for increased erythropoiesis (Krumidieck, 1943). Later on, in 1977, Epo protein was isolated form urine of anaemic patients (Miyake et al., 1977). Two groups independently succeeded in cloning the *EPO* gene in 1985 (Jacobs et al., 1985; Lin et al., 1985) and it is now clear that Epo is the essential link between reduced O_2 tension and increased Hct. The Epo-producing cells of the foetal liver and adult kidney are able to sense changes in tissue O_2 tension and respond with increasing *EPO* gene transcription. Epo is then secreted into the blood stream, reaches the sites of erythropoiesis in the bone marrow and stimulates proliferation and differentiation of the erythrocyte progenitors. Erythrocytes contain Hb, the protein responsible for O_2 binding and transport in the blood, thus an

increase in erythrocyte count restores the O_2-carrying capacity of the blood to normal levels (see figure 1) (Jelkmann, 2011; Wenger and Kurtz, 2011; Bunn, 2013).

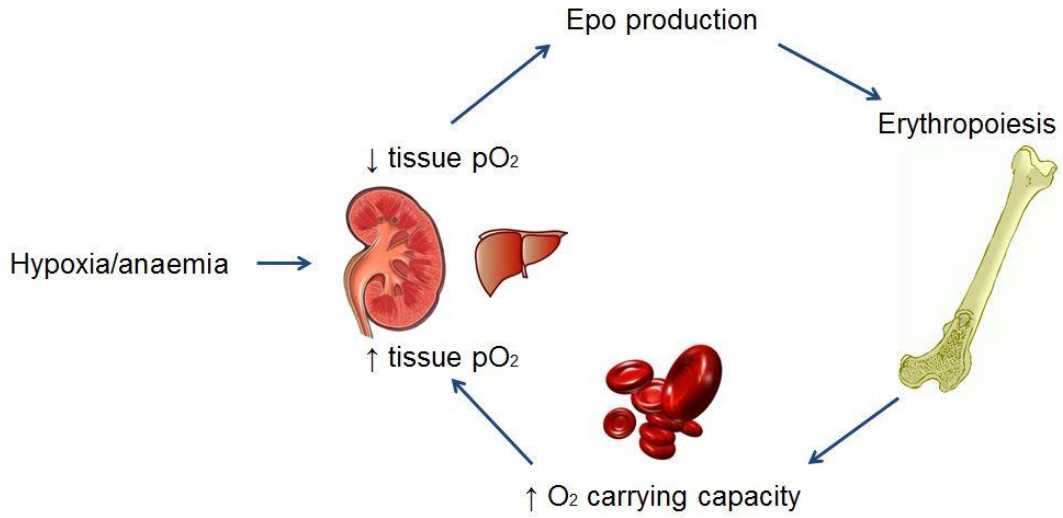

Figure 1 Scheme representing RBCs regulation by Epo.

The importance of Epo is demonstrated by animal experiments in which Epo or its receptor (EpoR) has been knocked out. $Epo^{-/-}$ or $EpoR^{-/-}$ mice showed the same phenotype with foetal death at embryonic day 12-13 (E12-13) associated with severe anaemia and the complete lack of definitive RBCs (Wu et al., 1995; Lin et al., 1996; Wu et al., 1999). On the other hand, many Epo transgenic mouse models have been generated and displayed erythrocytosis as a common phenotype (Semenza et al., 1989; Semenza et al., 1990; Loya et al., 1994; Madan et al., 1995; Köchling et al., 1998). Moreover, Epo amino acid sequence is strongly conserved in mammals, as well as the overall structure of the protein (Wen et al., 1993), supporting the relevance of Epo function and explaining interspecies cross-reactivity of the hormone.

Epo plasma concentration in healthy human beings is around 10 pM but this value can increase up to 1000-fold in certain environmental conditions, such as severe anaemia or hypoxia. The inverse relationship between Epo plasma levels and O_2 partial pressure/Hct/Hb concentration has been demonstrated in human beings exposed to high altitude hypoxia, as well as in several animal experiments using different ways to induce hypoxia or anaemia (figures 2A and 2B). Following a drop in alveolar and subsequently in arterial O_2 tension, plasma Epo levels start to rise after 60-90 minutes from the beginning of the hypoxic stimulus and continue to rise with a slope depending on the severity of the stimulus. The maximal peak is reached at 12-24 h in rodents (Abbrecht and Littell, 1972; Jelkmann, 1982) and around 48 h in humans (Abbrecht and Littell, 1972). Epo plasma

levels then decrease but remain higher than the baseline value if the hypoxic stimulus persists (figure 2C).

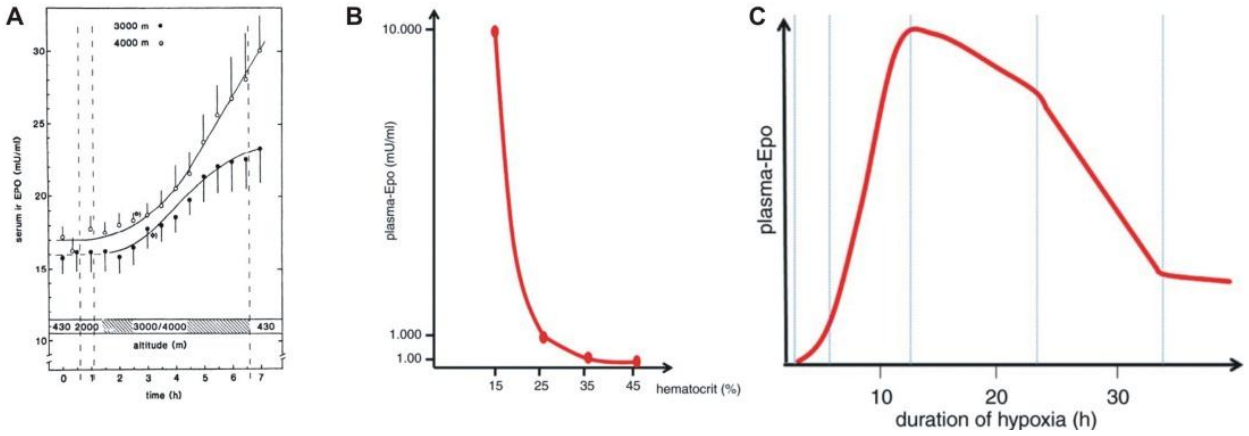

Figure 2 A. Time course of Epo serum plasma levels measured by radioimmunoassay (RIA) in human beings exposed to high altitude (hypobaric hypoxia) (Eckardt et al., 1989). **B.** Relationship between Hct and Epo plasma levels in anaemic rats (Hir et al., 1991). **C.** Time course of Epo plasma concentration in rats exposed to normobaric hypoxia (O_2 tension: 60 mmHg) (Wenger and Kurtz, 2011).

Since the Hct starts to rise long after the peak in Epo plasma levels (Abbrecht and Littell, 1972), it is reasonable to assume that an intrinsic feedback mechanism represses *EPO* transcription in Epo-producing cells before the effect of restoring the O_2-carrying capacity of the blood is actually achieved. Desensitization of Epo-producing cells to chronic hypoxia has been proposed as the underlying mechanism (see below).

Hypoxia is not the only stimulus capable of increasing Epo production: as mentioned above, all the conditions in which the O_2-carrying capacity of the blood is decreased lead to reduced tissue O_2 tension. This is sensed by the Epo-producing cells that respond with increased *EPO* transcription. Anaemia is defined as a reduction of the Hb content of the blood and can be due to blood loss or a variety of pathological conditions, such as chronic inflammation and tumour diseases (see below). With the exception of renal anaemia, where the main Epo-producing organ is affected, this condition is linked with increased Epo levels (Hir et al., 1991; Bunn, 2013). On the other hand, patients affected by primary polycythemia, a disease causing inappropriately increased erythropoiesis, display sub-normal Epo concentrations (figure 3) (Fernandez-Luna et al., 1998; Bunn, 2013). Differences in Hb affinity for oxygen affect O_2 release to the tissues and thus have an influence on Epo concentration as well: increased Hb affinity for O_2 leads to a rise in Epo plasma levels and *vice versa* (Lachermann, 1985). Of note, the curve of Epo level changes according to

Hct or Hb concentration has a different shape compared to the hypoxic curve: a fall in Hb concentration/Hct up to 70% of the normal value does not induce big changes in Epo levels, while after this threshold Epo plasma concentration increases exponentially (see figure 2B) (Cotes, 1982). This could be explained by the characteristic O_2-Hb dissociation curve, which allows sufficient oxygen delivery to peripheral tissues even with significant reduction of O_2-binding molecules (see below) (Boron and Boulpaep, 2005).

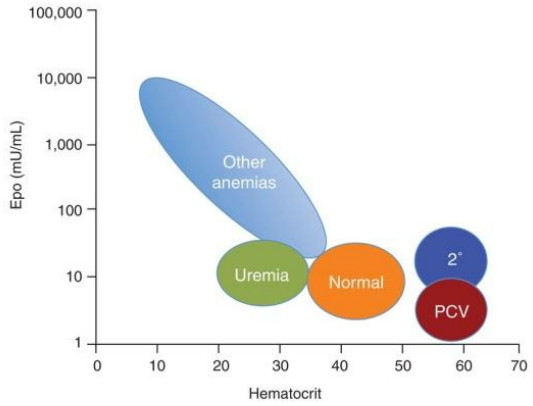

Figure 3 Plasma Epo levels measured in patients affected by different types and degrees of anaemia. Uremia = final stage of kidney failure (renal anaemia), PVC = *polycythemia vera* or primary erythrocytosis, 2° = secondary erythrocytosis (due to increased Epo levels, see below) (Bunn, 2013).

1.1.1 Sites of Epo production and Epo-expressing cellular models

Experiments with animals in which different organs have been surgically removed demonstrated the prominent role of the kidney in Epo production (Jacobson et al., 1957; Zanjani et al., 1981), confirmed by the fact that kidney diseases lead to anaemia due to impaired Epo synthesis (Wenger and Kurtz, 2011). In mammals, the kidney accounts for ~90% of total Epo production during adulthood, while the liver is considered the main source during foetal life (Caro et al., 1983; Clemons et al., 1986). The liver maintains its ability to produce Epo in response to hypoxia also after birth and represents the major site of extrarenal Epo secretion (Suzuki et al., 2013). Evidences in mice embryos suggest that Epo is synthetized by a sub-population of neural cells and neural crest cells in early-stage embryos (E8.5-11.5), before the liver starts supporting erythropoiesis in late-stage embryonic life (Suzuki et al., 2013). Epo has been detected in other organs, such as brain, lung, heart, bone marrow, spleen, hair follicles, the reproductive tract and osteoblasts, where it likely acts locally by regulating cellular viability (see below). The only

structural differences described for Epo protein produced by different organs concern the glycosylation pattern of the hormone (Masuda et al., 1994; Lönnberg et al., 2013).

The switch from liver to kidney occurs shortly after birth, in a species-dependent fashion. Studies in the ovine foetus revealed that both organs contribute significantly to Epo foetal levels and that a gradual change occurs from mainly hepatic to mainly renal Epo synthesis in late gestation (Moritz et al., 1997). The switch is found around postnatal day 10 in rats (Clemons et al., 1986), whereas it starts during the last trimester of gestation and ends several months after birth in humans (Dame and Juul, 2000). Differences in organ development are most likely the reason for the switch: in many species the kidney is not fully mature at birth and the oxygen-sensing capacity depends on intact and complete structure of the organ.

Liver

The fact that human Epo cDNA was first cloned from human foetal liver indicates the relevance of this organ for Epo production (Jacobs et al., 1985). The mammalian foetus is able to respond to a hypoxic stimulus by increasing Epo levels in the serum and amniotic fluid, as measured in sheep and rat foetuses (Clemons et al., 1986; Dame and Juul, 2000). The embryonic hypoxia-Epo axis is functional in human as well, since serum immunoreactive levels can be measured in blood samples from the umbilical cord starting at 18 weeks of gestation (Maxwell et al., 1994; Moritz et al., 1997). In spite of this, Epo levels in the foetus remain significantly lower than in adult mammals (Maxwell et al., 1994). The hepatic contribution to total Epo plasma levels in adulthood is considered to be around 10-20%, even though the extent seems to depend on the severity of the stimulus, suggesting that the liver is less sensitive to hypoxia compared to the kidney (Tan et al., 1991; Tan et al., 1992). Different O_2-sensing ability of the two organs is a potential explanation for the switch to the kidney after birth and is proven by the fact that the liver cannot compensate for a failure in renal Epo production. Another important difference between hepatic and renal Epo-producing cells concerns the mechanism of increased Epo synthesis in hypoxia: in the liver Epo mRNA seems to be up- and down-regulated in certain hepatocytes (Koury et al., 1991) while in the kidney a higher number of Epo-producing cells is recruited in case of hypoxia or anaemia (see below).

The cells responsible for O_2 sensing and Epo production in the liver are the hepatocytes themselves, as demonstrated at first by *in situ* hybridization using Epo antisense probe in liver of anaemic mice (Koury et al., 1991). Koury and colleagues also reported an increase in the number of Epo-positive hepatocytes around the central vein in mice carrying a human Epo transgene

construct. Many experiments with other transgenic mice carrying different Epo locus-driven constructs confirmed the ability of hepatocytes to express Epo (see below). Hepatic stellate cells, also known as Ito cells, can produce Epo too, as shown by a transgenic Epo-reporter mouse (Maxwell et al., 1994). Moreover, freshly isolated hepatocytes (Eckardt et al., 1993) and Ito cells (Maxwell et al., 1997) from normoxic animals are able to express Epo upon hypoxic exposure. Interestingly, Ito cells share some similarities with the renal Epo-producing cells, such as the expression of the fibroblast marker 5'-ectonucleotidase (Schmid et al., 1994).

Since attempts to isolate renal Epo-producing cells have failed so far, liver-derived cellular models have been historically used to study *EPO* gene regulation. The hepatocellular carcinoma cell lines Hep3B and HepG2 were shown to express Epo in an oxygen-dependent way already in 1987 by Goldberg et al. (Goldberg et al., 1987) and have been since then the most widely used *in vitro* models (Dame et al., 2004; Warnecke et al., 2004; Dame et al., 2006). In spite of this, Hep3B and HepG2 lack the typical feedback regulation of Epo mRNA under chronic hypoxia (see result section), as expected for the renal Epo-producing cells, and do not represent an appropriate model to study Epo regulation in the kidney. Another issue is the loss of hypoxia-induced Epo expression in these cell lines with increasing time in culture: cloning of the cell population is often required in order to maintain high Epo mRNA induction upon hypoxic exposure.

Kidney

As mentioned above, the kidney is known to be the most relevant Epo-producing organ. This observation fits with the kidney being the major oxygen-sensing organ in the body, together with the aortic and carotid body. The latter are located close to the lung circulation and are able to sense pO_2 and pH in arterial, freshly oxygenated blood. Since the tissue is highly perfused, the main function of the chemoreceptor cells in the aortic and carotid body is to regulate ventilation according to the pO_2/pH sensed in arterial blood by communicating with the respiration control centre of the brain stem. The location of the chemoreceptors does not allow them to sense changes in pO_2 arising from changes in the actual oxygen content of the blood, a function that is executed by the kidney (Boron and Boulpaep, 2005).

The kidney can be divided into an external part (cortex) and an internal part (medulla) and its primary function is to produce and excrete urine. The whole process of filtering the blood, reabsorbing and excreting electrolytes and substances according to the body needs is carried out by the epithelial cells of the tubular system. The nephron is the functional unit of the kidney and the urine produced in the lumen of each nephron converges in the pelvis and is excreted through the

ureters. This process is regulated in an extremely fine manner according to the blood volume, pressure and electrolytes/substances content. The vasculature is also peculiar in the kidney: the *vasa recta* in particular run parallel to the renal tubules within the medulla and, like the tubules, loop back up at various depths towards the cortico-medullary junction. As a result of the delicate anatomy of blood vessels, a countercurrent exchange of O_2 along the cortico-medullary axis takes place, leading to a unique gradient of O_2 towards the inner part of the organ, as well as to relatively low tissue O_2 tension. According to staining of rat kidneys with the hypoxic probe pimonidazole, the oxygen tension in the medulla is around 10-15 mmHg (Rosenberger et al., 2009), lower than what is found in other kidney regions where the range is 20-70 mmHg (Muller et al., 1998; O'Connor, 2006). It must be kept in mind that measuring kidney pO_2 directly is not easy, since insertion of electrodes (see below) can disrupt the architecture of the organ or result in broken electrodes: direct *in vivo* measurements are available only for the superficial cortex and show a high degree of variability (Evans et al., 2008). Moreover, the renal oxygen gradient is independent of changes in organ perfusion, since the more blood enters the kidney the more O_2 will be consumed by the tubular system, mainly for reabsorption, leading to a stable relationship between oxygen supply (blood perfusion) and consumption (see figure 4B) (Leong et al., 2007; Wenger and Hoogewijs, 2010). A relatively constant fraction of oxygen is consumed in the cortex, where filtration and a large part of reabsorption/secretion takes place, and renal Epo-producing cells (REPCs) are located at the border between cortex and medulla (see figure 4A), the perfect location to sense small changes in tissue O_2 tension arising from changes in the O_2 content of the blood.

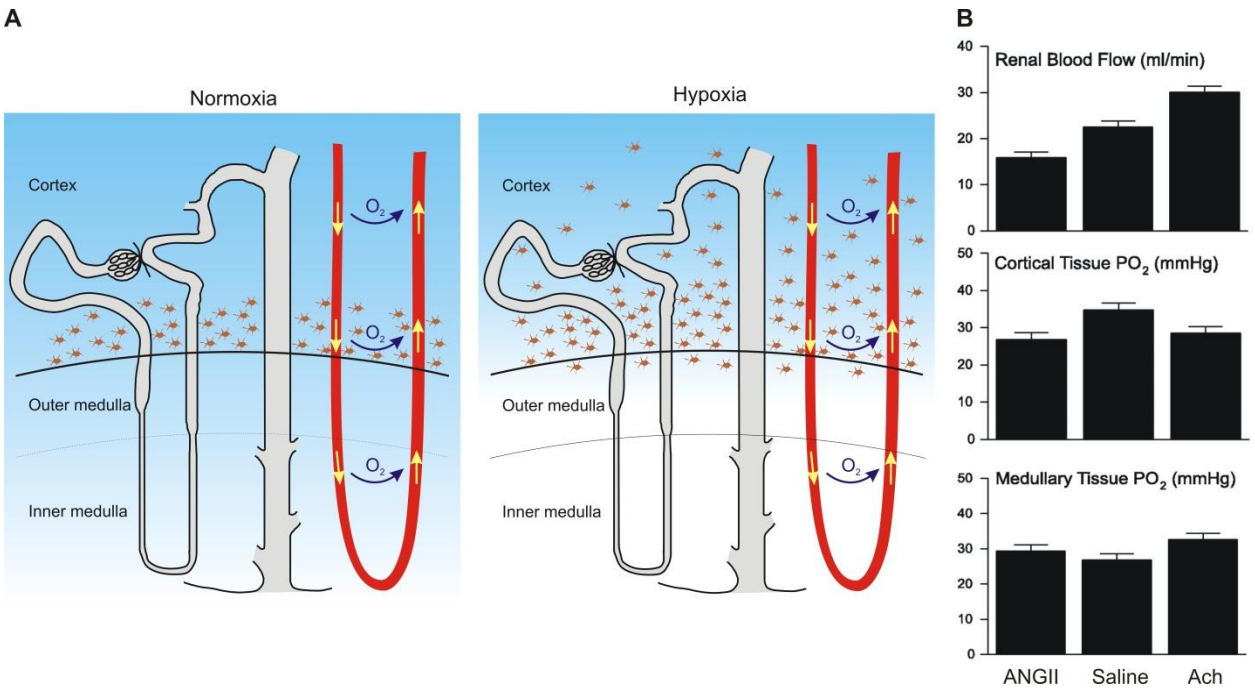

Figure 4 A. Schematic representation of the kidney tubular system and the countercurrent O_2 exchange leading to a stable cortico-medullary oxygen gradient (blue to white background). In hypoxia, the gradient is shifted to the outer cortex and the number of REPCs (brown cells) increases (adapted from (Wenger and Hoogewijs, 2010)). **B.** Changes in renal blood flow (RBF) were induced in rabbit kidneys by infusion of different stimuli in the renal artery (ANGII, Angiotensin II: decrease in RBF; Saline: no effect; Ach, Acetylcholine: increase in RBF). Cortical and medullary pO_2 remains relatively constant (Leong et al., 2007).

Since Epo is not stored in the cells that produce it, attempts to identify REPCs by immunohistochemistry did not provide reliable results. *In situ* hybridization represents instead a more suitable technique to study which renal cell types are responsible for Epo expression. In anaemic or hypoxic kidneys from different species (mice, rats, sheep and monkey), Epo mRNA is localized in interstitial, peritubular cells of the cortex and outer medulla (Koury et al., 1988; Lacombe et al., 1988; Eckardt et al., 1993; Darby et al., 1995; Fisher et al., 1996). The same expression pattern has been found in human near-term or postnatal kidneys from patients with conditions predisposing to severe hypoxia and/or induced polycythemia (Liapis et al., 1995). Epo-producing cells represent less than 10% of the total interstitial cell population of the kidney in severely anaemic animals and REPC number correlates directly with the amount of renal Epo mRNA and inversely with the Hct (Koury et al., 1989; Eckardt et al., 1993). Epo seems thus to be expressed in an all-or-none fashion and it is likely that an increasing number of Epo-producing cells is recruited depending on the severity of hypoxia or anaemia (see figure 4A). It is also

possible that the Epo expression pattern observed in these experiments is a consequence of the sensitivity of the technique, which would recognize positive cells only if they produce a “threshold” amount of Epo mRNA. Other studies made use of transgenic mice expressing human *EPO* locus-controlled green fluorescent protein (GFP) to localize REPCs and confirmed the results obtained by *in situ* hybridization. The group of Yamamoto in particular made use of transgenic mice generated with a bacterial artificial chromosome (BAC) containing 180 kb of the murine Epo gene flanking regions, which should encompass all the possible regulatory elements and allow accurate identification of REPCs (Obara et al., 2008; Suzuki et al., 2011). Even though the localization of REPCs is usually considered interstitial, it must be mentioned that the location of these cells has been a matter of debate for a long time. A number of reports in literature showed Epo mRNA expression in other cell types, in particular in the cortical proximal tubule, glomerulus and mesangium (Jelkmann et al., 1983; Kurtz et al., 1983; Mori et al., 1985; Loya et al., 1994; Fisher et al., 1996; Shanks et al., 1996). The stimuli and techniques used in these studies are usually different from the reports showing REPCs as interstitial cells: cobalt chloride ($CoCl_2$) or hypobaric hypoxia was used to mimic hypoxia in wild type (wt) mice or transgenic mice carrying LacZ reporter gene under the control of Epo locus were generated (Loya et al., 1994). In the last case, the flanking regulatory regions encompassing the transgene do not include the kidney-inducible element (see below). Nevertheless, tubular cells undergoing neoplastic transformation can acquire the ability to produce Epo, as demonstrated by Epo constitutive expression in different cell lines and human tumours originating from renal epithelium (Da Silva et al., 1990). A very recent report tried to solve the debate on REPC localization by using highly sensitive *in situ* hybridization techniques in normoxic and anaemic rats. Interestingly, in basal conditions Epo mRNA was detected mainly in tubular cells of the cortical collecting duct, whereas upon anaemia Epo expression became detectable in peritubular cells and only a slight increase could be observed in the tubular epithelium. These data suggest the possibility for Epo to be expressed by the renal tubule in normoxia but interstitial cells seem to be mainly responsible for the hypoxia-dependent increase in Epo concentration (Nagai et al., 2014).

Despite considerable research effort, the identity of renal Epo-producing cells remains not fully understood. Given the interstitial localization and the expression of markers such as the 5’-ectonucleotidase (CD73) and platelet-derived growth factor receptor β polypeptide (PDGF-β), REPCs were initially thought to be fibroblasts (Bachmann et al., 1993; Maxwell et al., 1993). An additional layer of complexity is caused by the observation that Epo-producing cells express markers of the neuronal lineage as well, including the microtubule-associated protein 2 (MAP2) and the neurofilament light polypeptide (NFL), and to display dendritic-like processes. No staining

for blood vessels (platelet endothelial cell adhesion molecule 1 or cluster of differentiation 31, PECAM/CD31) or macrophages (macrophage antigen 1, Mac1, and major histocompatibility complex II, MHCII) co-localized with REPCs (see figure 5) (Obara et al., 2008). The hypothesis of neuronal origin of REPCs is additionally supported by the fact that most of the interstitial fibroblasts in the kidney are derived from myelin protein 0 (P0)-positive neural crest cells and around 10% of P0 lineage-derived renal cells co-localize with Epo expression in transgenic mice (Asada et al., 2011). Moreover, a recent study identified neural crest cells as the source of Epo during embryonic development (Suzuki et al., 2013). Interestingly, renin-producing cells from the kidney juxtaglomerular apparatus have been shown to turn into Epo-producing cells when the von Hippel-Lindau protein (pVHL, see below) is knocked out, followed by HIF-2α stabilization. The study shows for the first time the possibility for this specific cell type to acquire REPC characteristics (Kurt et al., 2013). Even though from a different embryonic origin, renin-producing cell number also increases under low salt diet conditions, suggesting a parallelism between REPCs and renin-producing cells.

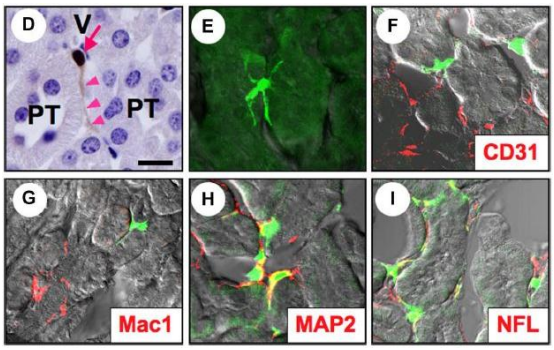

Figure 5 D/E. Epo-producing cells from an Epo locus-driven GFP transgenic mouse are indicated by arrows (interstitial localization). When co-stained with cell lineage markers (red), REPCs (green) were negative for CD31 (**F**) and Mac1 (**G**), but positive for MAP2 (**H**) and NFL (**I**) (Obara et al., 2008).

Attempts to isolate REPCs to obtain an *in vitro* kidney-derived model for hypoxic Epo regulation have been so far unsuccessful. Only one report in literature shows Epo production by E4, a mixed mesenchymal cell population derived from adult mouse kidney. E4 cells were able to up-regulate Epo mRNA and protein when exposed to anoxia or hypoxia and immunofluorescence experiments showed co-localization of Epo and the fibroblast marker CD73 in roughly 50% of the cells. Apart from the paper describing the cells, no independent study confirmed E4 as a renal cell model for Epo hypoxic regulation (Plotkin and Goligorsky, 2006). An Epo locus-driven GFP BAC was used to tag and isolate REPCs from anaemic mice: GFP-positive cells represented 0.2% of the total kidney

tissue and 67% of them co-expressed CD73 (Pan et al., 2011). However, isolated GFP-positive REPCs could not be kept in culture. Epo-producing cells are often considered fibroblasts in a “resting state” that lose the ability to produce Epo when induced to proliferate, as in case of *in vitro* culturing. Recently, a link between the loss of Epo production and differentiation of REPCs to myofibroblasts was proposed (Souma et al., 2013). In this study, a mouse model of renal fibrosis was used and the authors could show that most of the myofibroblasts governing fibrosis were derived from REPCs, which lost Epo expression after differentiation. Interestingly, the phenotype was rescued upon removal of the inflammatory stress. Conversion of REPCs to non Epo-producing fibroblasts would also explain anaemia in patients with CKD.

Other sites of Epo production and non-haematopoietic effects of Epo

As mentioned above, other cell types are capable of producing Epo, apparently in a similar hypoxic response like in liver and kidney. The functional relevance of these small amounts of Epo is probably a paracrine/autocrine protection from acute tissue injury. Epo receptor is indeed expressed by a number of tissues, such as brain (Tan et al., 1991; Dame et al., 2000), cardiomyocytes (Tramontano et al., 2003; Wright et al., 2004), megakaryocytes (Fraser et al., 1988), endothelial cells (Anagnostou et al., 1994; Yamaji et al., 1996), testis (Yamazaki et al., 2004) and cancer cells. In the case of tumours, the local production of Epo has been proposed to act directly on EpoR of the same neoplastic tissue and to promote proliferation, invasion and chemoresistance (a few of the most recent examples include (Wu et al., 2012; Rózsás et al., 2013; Todaro et al., 2013; Liang et al., 2014)).

The brain is one of the most studied alternative Epo-producing tissues: expression of Epo and its receptor has been shown in neurons, astrocytes and endothelial cells throughout development (Tan et al., 1991; Dame et al., 2000) and conditional knockout of EpoR in brain impairs neural cell proliferation and post-stroke neurogenesis (Tsai et al., 2006). Astrocytes in particular are capable of oxygen-regulated Epo expression *in vitro* (Masuda et al., 1994) and *in vivo* (Tan et al., 1991). In the retina, Epo and EpoR have been found to be expressed by the optical nerve, retinal ganglion cells, photoreceptors, retinal pigmental and vascular cells, suggesting a role for the hormone in this tissue (Juul et al., 1998; Hu et al., 2011; Caprara et al., 2014). Also, during development and pathological conditions such as diabetic retinopathy Epo retinal levels are significantly increased (Xie et al., 2007; García-Ramírez et al., 2008). Again, a protective role for Epo on retinal neuron has been proposed by many groups (reviewed by (Grimm et al., 2006)). Interestingly, the brain stem, that is responsible for respiration control, seems to express EpoR, as the chemoreceptors of the carotid body, which have also been shown to be a site of Epo production. This suggests the

possibility of a coordinated physiological adaptation to hypoxia in which Epo would interact directly with the respiration control centres (Soliz et al., 2005; Lam et al., 2009; Soliz et al., 2009). Epo expression in the brain is supported by the fact that neuronal cell models are capable of expressing oxygen-dependent Epo in culture. The neuroblastoma cell lines Kelly and SH-SY5Y in particular are used as *in vitro* model for hypoxic Epo expression (see results) (Stolze et al., 2002).

Evidence exists for a protective role for Epo after tissue damage in the heart as well. Several groups reported beneficial effect of recombinant Epo in animal models of ischemia/reperfusion and myocardial infarction (Parsa et al., 2004; Fiordaliso et al., 2005; Nishiya et al., 2006). The mechanism underlying Epo cytoprotective effect is probably link to the pro-survival and anti-apoptotic JAK/STAT pathway activated upon interaction of Epo with its receptor (see below). Another proposed explanation is the effect of Epo on endothelial cells and angiogenesis, also thought to be beneficial for tumour cells invasiveness (Wu et al., 2012; Liang et al., 2014). High doses of rhEpo have been shown to stimulate endothelial cell proliferation *in vitro* (Anagnostou et al., 1990) and systemic hypoxia can mobilize $CD34^+$ endothelial progenitor cells in an Epo-EpoR-dependent manner *in vivo* (Bahlmann et al., 2003; Heeschen et al., 2003). On the other hand, angiogenesis in skeletal muscle seems independent from endogenous Epo (Hagström et al., 2010). The two main Epo-producing organs, the liver and the kidney, have also been shown to express EpoR and to be protected by Epo administration in a series of tissue injury model (Cassis et al., 2011; de Souza et al., 2012; Xiao et al., 2012; Fu et al., 2013).

The physiological relevance of the above described Epo non-haematopoietic effects is still debated. Epo/EpoR KO mice do show cardiac defects, abnormal vascular development and increased apoptosis in the brain besides failure in definitive erythropoiesis (Wu et al., 1999). When EpoR was specifically re-introduced in the erythroid lineage, mice survived and showed no apparent developmental defect (Suzuki et al., 2002). Additionally, a few reports in literature support the lack of functionality of the Epo-EpoR axis in tissues other than the hematopoietic compartment due to much lower expression of EpoR or to unresponsiveness of the intracellular signalling pathway when rhEpo was administered *in vitro* (Sinclair et al., 2010; Elliott et al., 2012).

1.1.2 Erythropoietic effect of Epo

Epo structure and binding to EpoR

Once Epo is secreted from REPCs into the blood stream, it has to reach the sites of haematopoiesis in the foetal liver and adult bone marrow in order to activate RBC production. Epo protein is a ~30.4 kDa, heavily glycosylated hormone. Evidences suggest the appearance of Epo

already in fish, such as zebrafish and trout (Chu et al., 2008), but most of the knowledge we have comes from studies in mammals that show strong interspecies conservation in terms of amino acid sequence and structure (Wen et al., 1993). Epo precursor protein contains a canonical leader sequence and is cleaved in the endoplasmic reticulum, glycosylated in the Golgi and finally released as a 166 residues polypeptide (Jacobs et al., 1985; Lin et al., 1985). Epo folds as a globular protein composed by 4 α-helices connected by loops and stabilized by two disulphide bridges (Cheetham et al., 1998; Syed et al., 1998). One of the disulphide bridges is critical for the activity of the protein and links the amino-terminus with the carboxy-terminus of the polypeptide generating a long loop, probably important for binding to EpoR (Boissel et al., 1993). Glycosylation of the human protein consists of three *N*-linked polysaccharide groups and one *O*-linked group, accounting for roughly 40% of total Epo weight (Sasaki et al., 1988). The polysaccharide groups are needed to increase Epo plasma half-life but are probably dispensable for biological activity: removal of terminal sialic acids increases Epo activity *in vitro* but shortens the half-life of the polypeptide from 7-8 h to a few minutes *in vivo* (Goldwasser et al., 1974). The different types of commercially available rhEpo (Epoetin alpha, beta, delta, omega and zeta) differ indeed in the glycosylation residues composition.

Epo binds to the high affinity EpoR present on the surface of target cells, mainly erythrocytes precursors. EpoR is a 70-80 kDa protein containing post-translational glycosylations and phosphorylations (Sawyer and Hankins, 1993). It belongs to the cytokine receptor superfamily and consists of homodimers of single transmembrane monomer associated to the Janus kinase family member 2 (JAK2) (Ihle, 1995). Upon Epo binding, the two subunits of EpoR homodimer are brought in close proximity and so are the JAK2 domains: this conformational change initiates the transduction cascade by enabling cross-phosphorylation of JAK2 and phosphorylation of the cytosolic tail of EpoR subunits. The newly generated SH2 docking sites recruit several proteins, including the signal transducer and activator of transcription (STAT)-5, which upon phosphorylation homodimerizes, translocates to the nucleus and activates a series of target genes leading to anti-apoptotic, pro-survival and pro-erythrocyte differentiation effects (Watowich et al., 1994). Other intracellular signalling pathway activated by Epo-EpoR interaction include the phosphatidylinositol 3 kinase (PI3K)/Akt, the extracellular-regulated kinases 1/2 (ERK1/2) and p38 pathways (Richmond et al., 2005). JAK2 dephosphorylation and receptor internalization then terminate EpoR signalling. The essential role of JAK2 in erythropoiesis is proven by experiments with *Jak2*$^{-/-}$ mice, which are anaemic and die at embryonic day 12.5 due to lack of definitive erythropoiesis (Neubauer et al., 1998) .

Primitive and definitive erythropoiesis

Erythropoiesis (from Greek “erythro” = red and “poiesis” = to make) is the process by which RBCs are produced. Self-renewing properties of haematopoietic stem cells provide a continuous replenishment of differentiated erythrocytes (Orkin, 2000). A first wave of erythrocyte synthesis originates in the “blood islands” within the yolk sack and is known as primitive erythropoiesis. This early foetal life population consists of large, nucleated cells and their production takes place between embryonic day 7 and 9 in mice (Palis et al., 1999). The role of Epo in primitive erythropoiesis is still not well defined but recent studies in EpoR-null embryos revealed the importance of Epo-EpoR signalling pathway for primitive erythrocyte maturation (Malik et al., 2013). The finding of Epo expression in neural crest cells by Suzuki et al. also supports the idea of Epo acting already on erythropoiesis at this early stage (Suzuki et al., 2013).

Definitive erythropoiesis occurs instead in the foetal liver and postnatal bone marrow, where the whole haematopoiesis, the production of blood cells, takes place starting from a common pluripotent stem cell. Figure 6 illustrates the different cell lineages arising from the self-renewing stem cell, including the erythropoietic lineage (highlighted on the left). Stem cell progeny is divided into committed stem cells, which continue with differentiation, and pluripotent stem cells, which maintain the self-renewing pool. Cell division of the committed progenitor originates the myeloid lineage on one hand, to which RBCs belong together with platelets, granulocytes, mast cells, monocytes and dendritic cells, and the lymphoid lineage on the other hand, including B-, T- and NK-cells (Stirewalt and Radich, 2003).

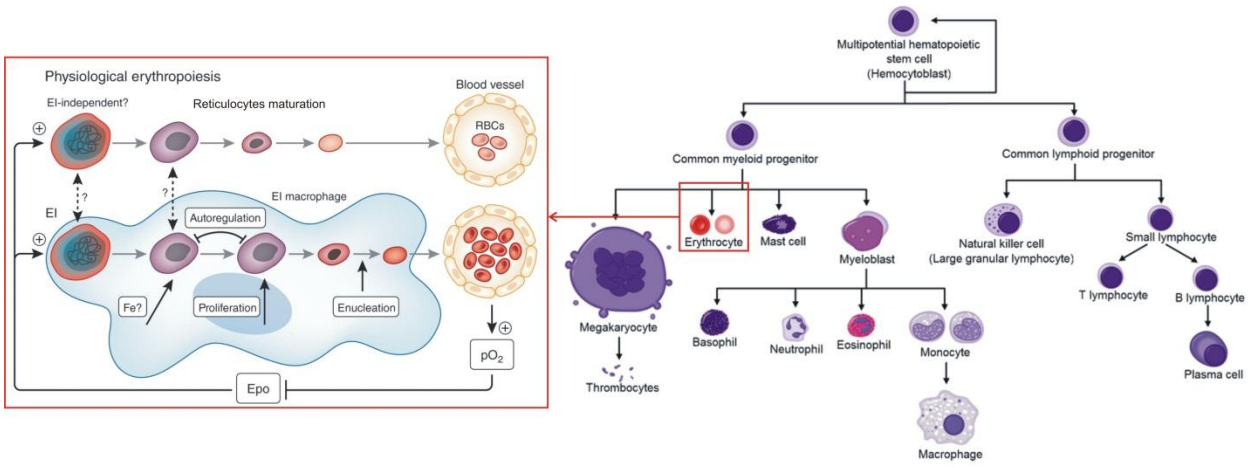

Figure 6 Right panel: schematic representation of the haematopoietic process with the various blood cell lineages deriving from the common stem cell. The erythroid differentiation line is highlighted in red. Left panel: physiological definitive erythropoiesis within and without

erythroblastic island (EI). Macrophages within the EI support eytrhoblasts by promoting cell proliferation, assisting iron uptake and enucleation and keeping cells in close proximity in order to allow autoregulatory inetractions. Dashed arrows indicate the possibility for erythroblasts to repeatedly attach and detach from the EI. Regulation of Epo production by RBC amount in the blood is shown (adapted from (Stirewalt and Radich, 2003; Socolovsky, 2013)).

The most immature compartment of definitive erythroid progenitors consist of the burst-forming unit-erythroid (BFU-E). When kept in culture, human BFU-E take 14 days to form colonies of mature erythrocytes in semisolid media. The next step is represented by the colony-forming unit-erythroid (CFU-E), that take only 7 days to give mature erythrocytes *in vitro*, suggesting a more committed phenotype compared to BFU-E (Palis et al., 1999). Epo is essential for CFU-E survival and required for colonies formation in culture conditions (Koury and Bondurant, 1990; Wickrema et al., 1992). *In vivo*, CFU-E progenitor cells proliferate and differentiate into progressive erythroblast stages during which chromatin is condensed (nuclear pyknosis), cell size is decreased, haemoglobin is accumulated (iron-dependent phase) and RNA content is diminished (Koury et al., 1989; Liu et al., 2011). The final, fundamental step in RBC maturation is enucleation: the newly formed erythrocytes, also called reticulocytes, lose the nucleus and all the residual cytoplasmatic organelles (especially mitochondria), reduce cell volume and plasma membrane surface and are then released into the blood stream (Palis et al., 1999; Ji et al., 2011). The typical shape of mature RBCs is a biconcave disc. Erythroblasts are able to generate mature RBCs *in vitro* without the need of accessory cell types but this is probably not valid for the haematopoietic tissues, where specific niches called erythroblastic islands (EI) can be found. An EI consists of one or more central macrophages to which differentiating erythrobalsts attach in concentric rings. EIs are thought to have crucial support functions to the whole process of RBC maturation, mainly by promoting cell proliferation, iron-uptake and cell-cell interaction by secreting paracrine factor and keeping cells in close proximity. The enucleation step is also facilitated by the presence of EIs (Manwani and Bieker, 2008; Mohandas and Chasis, 2010).

Erythrocyte function and haemoglobin

It has been calculated that an average adult with approximately 5 L of blood renews more than 10^{11} reticulocytes per day (~1% of total) in order to keep a roughly constant number of RBCs (Lang et al., 2012; Palis, 2014). Mature erythrocytes feature unique cytoskeletal characteristics that allow them to repeatedly and passively deform when passing though narrow capillaries. Plasma membrane is for example attached to an elastic network of spectrin and actin cytoskeleton through a series or protein, including glycophorin C and ankyrin band 3. Mutations in the genes encoding

for such proteins lead to severe haemolytic disorders (characterized by erythrocyte lysis, reviewed by (Mohandas and Gallagher, 2008)).

As mentioned above, oxygen solubility in plasma is not sufficient to transport an adequate amount of gas to the whole organism. The most important protein involved in O_2 transport in the blood is haemoglobin, which is also the most abundant protein in RBCs. One molecule of adult Hb contains 2 α and 2 β chains, each consisting of a globin polypeptide and a heme group, the binding site of molecular oxygen. The porphyrin ring of the heme group chelates a metal atom, iron in the ferrous state (Fe^{2+}) in the case of Hb. The interaction between O_2 and iron leads to conformational changes in the protein tri-dimensional structure that facilitates the binding of additional oxygen molecules to the other binding sites of Hb. This cooperative effect between the 4 binding sites is responsible for the typical S-shape of the Hb-O_2 dissociation curve (figure 7) and allows easier load and unload of the protein according to pO_2. Several factors can cause allosteric changes in Hb conformation and consequently a shift of the curve towards right or left, i.e. decreased or increased affinity for oxygen. Acidic pH, increased carbon dioxide partial pressure (pCO_2) and higher temperature shift the curve to the right, while basic pH, decreased pCO_2 and lower temperature contribute to the leftward shift of the curve. Physiologically, these effects are strongly relevant because they allow easier O_2 dissociation (and thus increased O_2 availability) in metabolically active tissues, such as skeletal muscles where pH is low and pCO_2 and temperature are high. On the other hand easier O_2 binding in metabolically inactive tissue, such as the lungs, is ensured. The affinity of Hb for oxygen is also sensitive to the concentration of 2,3-diphosphoglycerate (2,3-DPG), a metabolite of glycolysis that binds in 1:1 ratio to Hb molecules. Binding of 2,3-DPG to Hb destabilizes the interaction of the protein with oxygen. A physiologically relevant situation such as hypoxia leads to a switch to glycolytic metabolism, thereby increasing 2,3-DPG content and facilitating O_2 release to the tissues (Boron and Boulpaep, 2005; Mairbäurl and Weber, 2011).

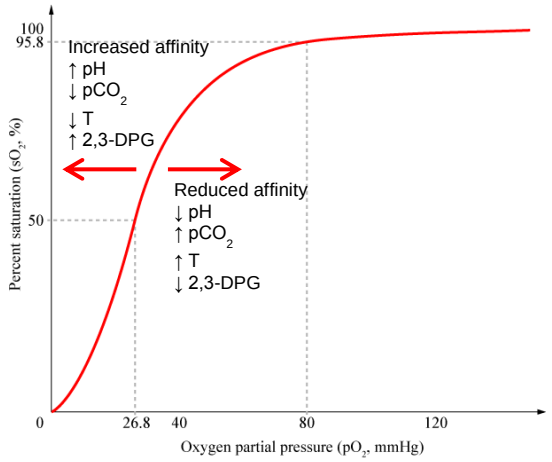

Figure 7 Oxygen-haemoglobin dissociation curve: pO_2 in arterial blood is around 100 mmHg and the saturation of Hb in physiological conditions is ~97.5%, pO_2 in mixed venous blood is around 40 mmHg and Hb saturation is ~75%. The factors increasing or decreasing the affinity of the protein are shown.

It is important to mention that different forms of Hb are expressed in embryonic and foetal life, due to the different way by which oxygen is delivered to the mammalian embryo/foetus. Maternal blood is the main source of O_2 and increased Hb affinity is required for the embryo in order to get enough oxygenation. Additional globin chains build the embryonic and foetal forms of Hb and include α-like chains (ζ) and β-like chains (γ, δ and ε), encoded by different genes in the same chromosomal loci (chromosome 16 for α-like and 11 for β-like chains in the human genome). At birth, the newborn blood contains both adult and foetal Hb but the latter gradually decreases till 1-2% of the total (Wild and Bain, 2004). Obviously, mutations in Hb-coding gens lead to various forms of anaemia, among which sickle cell anaemia is one of the most clinically relevant. A single amino acid replacement (V6E) renders the de-oxygenated form of sickle Hb much less soluble: the result is precipitation of the protein inside RBCs and formation of long fibres, giving the typical sickle-like appearance. Besides being less efficient in carrying oxygen, sickle cells are also more prone to haemolysis (Mairbäurl and Weber, 2011).

RBCs are fundamental not only for oxygen transport in the blood but for CO_2 carriage as well. Most of the CO_2 (90% of total) is in fact transported inside erythrocytes in different forms: a small fraction is dissolved (4%), about 22% reacts with Hb forming carbamino groups and the other 64% is converted to bicarbonate (HCO_3^-) via carbonic anhydrases in the cytosol of RBCs. HCO_3^- is then transported out of the cells via the chloride-bicarbonate exchanger AE-1 (anion exchanger 1) (Boron and Boulpaep, 2005).

The average life of erythrocytes in the blood is of 100-120 days. As RBCs age, the size and cytoplasm volume get smaller, while Hb concentration remains stable, resulting in increased density. Macrophages of the phagocyte system in the spleen, liver and lymph nodes are able to recognize senescent erythrocytes thanks to a series of changes in the composition of the plasma membrane. During senescence, modified Hb binds to band 3 leading to modification of the protein, IgG binding and dissociation of the cytoskeleton from the lipid bilayer. Moreover, vesicles exposing senescence markers and phosphatidylserine are fused to the plasma membrane, making the "old" RBC a target for scavenger receptors expressed by macrophages (Arese et al., 2005; Bosman et al., 2005; Lang et al., 2010). An additional mechanism reducing the amount of damaged RBCs is eryptosis or suicidal death of erythrocytes, which may occur in case of cell injury prior to senescence. Eryptosis is a kind of programmed cell death, similar to apoptosis in nucleated cells, and involves calcium influx into the erythrocyte. In contrast to haemolysis, there is no rupture of the cell membrane and no release of the intracellular content but rather blebbing and exposure of removal signal, such as phosphatidylserine (Lang et al., 2010; Lang et al., 2011). Interestingly, Epo has been shown to inhibit suicidal death of RBCs (Myssina et al., 2003). The physiological significance of eryptosis could be removal of defective erythrocytes and prevention of haemolysis, a necrosis-like process leading to disruption of the cell membrane and release of Hb in the blood stream. Hb is filtered in the kidney and big amounts of circulating protein can have serious consequences, such as renal tubule occlusion.

1.2 Hypoxia

Oxygen is essential for survival of nearly all plants, animals and single-cell organisms, thus its concentration in tissues must be maintained within a certain range in order to avoid conditions of hypoxia (not enough O_2) or hyperoxia (too much O_2), both harmful for aerobic cells. Evolution led to generation of organisms (plants) that are able to convert solar energy, CO_2 and H_2O into energy-containing glucose molecules through the process of photosynthesis, with the production of O_2 molecules as a side product. The increased oxygen levels in the atmosphere was followed by the evolution of multicellular organisms (*Metazoa*) metabolizing glucose in an oxygen-dependent way in order to extract energy, via the so-called aerobic respiration taking place in special cellular organelles, the mitochondria. This process is extremely efficient, with a virtual yield of 30 ATP molecules per glucose molecule metabolized through glycolysis and citric acid cycle. Anaerobic glycolysis alone leads to the production of 2 ATP molecules/glucose mole while β-oxidation of fatty acids gives around 14 ATPs/glucose mole consumed (Alberts et al., 2002; Semenza, 2014).

Sensing and maintaining O_2 homeostasis is not only a task of tissues directly involved in O_2 uptake and delivery, such as the aortic and carotid body, the vascular and respiratory system, but of all the nucleated cells of our body that are able to respond to hypoxia by activating the ubiquitous von Hippel-Lindau protein/prolyl-4-hydroxylase domain/hypoxia-inducible factor (pVHL/PHD/HIF) pathway, explained in details below.

What is the real partial pressure of oxygen cells are exposed to inside our body? And what does hypoxia mean from this point of view? Considering hypoxia every O_2 concentration values below 21% or every pO_2 below 160 mmHg (the fraction of O_2 in the air at sea level) would be a mistake since most of the cells of the organism are actually exposed to much lower oxygen tension, as summarized in table 1 (Carreau et al., 2011). Already from external air to air in the alveoli, there is a significant drop in oxygen tension of 50 mmHg and this value further decreases when considering arterial, freshly oxygenated blood. Different tissues have specific pO_2 depending on a number of factors, such as blood perfusion, vascular anatomy and O_2 consumption of the organ. As mentioned above, the kidney features a unique, wide oxygen gradient with values ranging from 10-20 mmHg in the medulla to 50-70 mmHg in the cortex. Hypoxia is thus a “functional” definition: hypoxic cells are exposed to an oxygen tension that is lower than the physiological one for a given tissue and that triggers the activation of hypoxia-responsive pathways. The lowest oxygen partial pressure at which mitochondria can still perform aerobic respiration is thought to be around 0.15-0.3 kPa (1-2 mmHg), the so-called Pasteur point (Sandberg and Naylor, 2011). It is interesting to notice that *in vitro* cell cultures are usually performed in 95% air (+ 5% CO_2), thus providing 19.95% O_2 (~ 151.7 mmHg), a concentration that probably results in hyperoxia compared to physiological conditions inside the human body.

Compartment/Tissue	pO_2 (mmHg)
Air	160
Inspired air (tracheus)	150
Air in the alveoli	110
Arterial blood	100
Venous blood	40
Brain	33.8 ± 2.6
Lung	42.8
Liver	40.6 ± 5.4
Kidney cortex	72.0 ± 20
Kidney medulla	10 - 20
Muscle	29.2 ± 1.8

Table 1 Mean oxygen tension values in the microenvironment of different human tissues (adapted from (Carreau et al., 2011)).

The most direct way to measure oxygen levels in tissues is represented by polarographic sensors. This method is based on the classical Clark's electrodes, consisting of a noble material (e.g. silver, gold or platinum) which reduces oxygen thanks to a negative polarizing voltage. The measurement takes into account the difference between a reference electrode (anode) and a measuring one (cathode) and it is directly proportional to the amount of oxygen reduced by the cathode. The tip of the electrode is very thin (3-5 μm) and electrically insulated by a gas-permeable but liquid-impermeable membrane. Inserting the electrodes into the analysed tissue has been so far considered as the standard procedure (Clark et al., 1958; Clark et al., 1958; Norton and Rand, 1974). Optic fibre-based sensors represent another option and are based on O_2-dependent changes in the lifetime of the pulse of a fluorescent dye placed at the tip of the sensor. The advantage compared to the Clark's electrodes is that oxygen is not consumed by this reaction, so that measurements can be recorded continuously (Vanderkooi et al., 1987; Griffiths and Robinson, 1999). Additionally, a few groups have used mass spectrometry in order to quantify molecular oxygen in tissue samples (Ndubuizu and LaManna, 2007). Given the invasiveness of the described methods, different techniques to visualize hypoxic regions are used in the clinics, for example for tumour imaging. Pimonidazole and other nitroimidazole compounds are the gold standard "exogenous" hypoxic markers, since they require immunohistochemical methods to be developed. Pimonidazole is selectively reduced in hypoxic conditions to form hydroxylamine that irreversibly

binds to proteins or DNA in the cells. The signal dramatically increases below 10 mmHg of oxygen and the probe can also be injected into the patient prior to biopsy of the tumour (Nunn et al., 1995). Non-invasive methods include PET (positron emission tomography), NIRS (near-infrared spectroscopy) and MRS (magnetic resonance spectroscopy), which do not measure directly oxygen tension but rather target hypoxic areas by imaging oxygenation of the blood or O_2 consumption in the tissue (reviewed by (Carreau et al., 2011)).

The mammalian response or adaptation to hypoxia can be considered at the systemic and at the cellular level. The systemic response occurs when the entire organism is exposed to low pO_2, as in the case of high altitude, and includes a series of changes involving mainly the respiratory and vascular systems. The cellular response studies the activation of oxygen-sensitive pathways within the cell, with the stabilization of the transcription factors of the HIF family as central mechanism, and can be localized to a specific tissue/group of cells. The two levels are certainly connected; in particular the activation of the cellular pVHL/PHD/HIF pathway underlies adaptation to systemic chronic hypoxia.

1.2.1 Systemic response to hypoxia: adaptation to high altitude

When describing adaptation to high altitude, we refer to the so-called hypobaric hypoxia, which occurs when the pO_2 is lower due to the lower total pressure of the air gases mix. The higher we go, the lower total air pressure and, subsequently, the lower pO_2. Air pressure is considered to fall by around half every 5500 m (Boron and Boulpaep, 2005). Usually, an altitude of 1500-2500 m is known as “moderate”, while an altitude of > 2500 m is considered as “high”, since the pO_2 falls low enough to cause a systemic adaptive response in human beings, sometimes leading to acute mountain sickness (AMS) (Hill et al., 2011).

As mentioned above, the aortic and carotid bodies are responsible for sensing pO_2 in arterial, freshly oxygenated blood. These highly vascularized organs contain chemoreceptor cells (Type I or glomus cells) able to sense changes in oxygen tension, as well as in blood pH, which is in direct relation with pCO_2. Of note, peripheral chemoreceptors cannot sense changes in oxygen delivery arising, for instance, from anaemia. In case of hypoxia, acidosis and hypercapnia (increased pCO_2), the K^+ channels in the membrane of glomus cells are inhibited, voltage-sensitive Ca_2^+ channels open, intracellular Ca_2^+ increases and neurotransmitters are released. The threshold after which the transmitter release is significantly increased is around 60 mmHg of pO_2 in arterial blood, which corresponds to roughly 40% of reduction compared to physiological conditions (Lahiria et al., 2006). The mechanisms behind K^+ channel inhibition by hypoxia in glomus cells are not fully

understood. Involvement of protein kinase C (PKC), NADPH oxidase (NOX) and reactive oxygen species (ROS) has been demonstrated *in vitro* but this pathway is not driving the complete response *in vivo*, since NOX inhibition is not sufficient to suppress the hypoxia-mediated effect (O'Kelly et al., 2001; Buttigieg et al., 2012). The nerve fibres of peripheral chemoreceptors project in the cardiorespiratory centre of the medulla, leading to an acute and reversible increase in ventilation in order to reduce pCO_2 and maximize the uptake of O_2. Interestingly, this acute increase in ventilation is followed by a ventilatory decline, probably when CO_2 levels fall too low, and by a slow increase in ventilation if the hypoxic stimulus persists (Boron and Boulpaep, 2005; Sandberg and Naylor, 2011). This slow increase occurs during the acclimatization phase, usually during the next few days to weeks at high altitude, and is mediated by central chemoreceptors sensing a decrease in the pH of the cerebral-spinal fluid. The kidney also accounts for acclimatization by decreasing acid secretion to counteract respiratory alkalosis due to the initial hyperventilation (Boron and Boulpaep, 2005).

Changes in the respiration pattern are paralleled by changes in cardiac output and heart rate: the two parameters acutely increase in order to deliver more oxygen to peripheral tissues. The reason for this effect is the increase in sympathetic activity during hyperventilation, due both to activation of the lung stretch receptors and of the central chemoreceptors responding to decreased pCO_2. Acclimatized subjects and natives of high altitude display normal cardiac output and heart rate values, suggesting that adaptation has occurred (Sandberg and Naylor, 2011). Additionally, hypoxia has a direct effect on smooth muscle cells regulating blood vessel resistance. Local hypoxia in the alveoli inhibits K^+ channels, in a similar way as in the glomus cells, resulting in Ca_2^+ release from the intracellular storages and extracellular space and, ultimately, in myocyte contraction. Hypoxic pulmonary vasoconstriction is thought to be a residual mechanism from birth, when the foetal circulation transforms into the newborn one. Increased resistance in lung capillary can cause pulmonary hypertension and consequently right ventricular hypertrophy (Hooper and Mellor, 2011). In around 5% of low landers going to high altitude over a short period of time, pulmonary hypertension leads to pulmonary oedema, one the hallmarks of severe AMS. The incidence of this condition increases with the degree of altitude (Basnyat et al., 2000). On the other hand, cerebral blood flow is increased, probably to ensure sufficient oxygen delivery to a demanding organ like the brain. The effect seems to follow carotid and cerebral vessel vasodilation and can cause headache in non-acclimatized subjects (Wolff, 2000). The molecular mechanisms underlying changes in vascular tone are not completely understood but the central and peripheral chemoreceptors play certainly a fundamental role in the process of adaptation.

Stimulation of the carotid body by hypoxia additionally leads to natriuresis and diuresis, with an acute effect of reducing plasma volume (Honig, 1989). Another reason for decreased plasma volume could be dehydration due to evaporative loss during profound respiration in the first hours/days in hypoxia. However, the most important haematological change is the increase in red cell mass as a consequence of Epo production, which occurs via the classical HIF pathway activation in renal Epo-producing cells (see below). It has been calculated that, when corrected for loss of body weight, there is a mean increase of 67.5% in RBC mass with a final increase in plasma volume of 22.8% after a few days/weeks at high altitude (Hooper and Mellor, 2011). Increased incidence of thrombosis in individuals ascending to high altitude can be explained by both pulmonary vasoconstriction and increased blood viscosity due to higher count of RBCs (Gupta and Ashraf, 2012).

AMS is a clinical condition occurring upon hypoxic exposure of non-acclimatized individuals going to high altitude, with an incidence of ~10% for those going from sea level to 2500 m and of 30% to 40% when ascending to 3500 m or more (Mairer et al., 2009). It develops within 1 to 5 days during the ascent and the main symptoms include fatigue, dyspnoea (shortness of breath), sleep disturbance, headache, dizziness and nausea. The hyperventilatory response explains the dyspnoea and sense of fatigue and causes during the night alternative periods of respiratory stimulation by hypoxia followed by apneas or hypopneas due to inhibition by hyperventilation-induced hypocapnia (Weil, 2004). Headache is probably the most common symptom of acute high altitude illness and is thought to derive from vasodilation of cerebral vessels and brain swelling. Cases of cerebral oedema have been reported and represent the most dangerous, life-threatening situation (Wilson et al., 2009). As mentioned above, pulmonary oedema can also occur. The best treatment for AMS consists in descending to lower altitude or in giving oxygen to the patient in order to remove the primary cause of the symptoms, hypoxia. Pre-acclimatization, slow ascent and fluid intake are all protective factors against AMS, while drugs such as antihypertensive, analgesic and beta-blockers are used to treat severe symptoms (Netzer et al., 2013). The term CMS (chronic mountain sickness) refers instead to the overproduction of RBCs observed in some individuals exposed to prolonged periods of high altitude. Polycythemia is thus the primary cause of this clinical condition, characterized by increased risk of thrombosis and right ventricular hypertrophy due to increased blood viscosity and pulmonary vasoconstriction (Boron and Boulpaep, 2005).

Given all the consequences that exposure to hypobaric hypoxia can have, how do populations like Tibetans and Andeans constantly live at 3000-5000 m of altitude? It is interesting to notice that the two populations took two different ways to adapt to the extreme environmental conditions found at high altitude, both successfully. Starting from ventilation, Tibetans seem to have kept the

hyperventilatory response typical of low landers going to high altitude, as demonstrated by their increase in resting ventilation, while Andeans show sea level-like or even lower ventilation rates (Beall et al., 1997). Hb concentration is surprisingly lower in Tibetans compared to Andeans living at the same altitude and is accompanied by lower Epo levels and O_2 saturation of the blood, making Tibetan people profoundly hypoxic (see figure 8) (Beall et al., 1998). People living in the Andes often display pulmonary hypertension, whereas Tibetans do not show any sign of lung capillary vasoconstriction. Also, the response to pregnancy is different in the two representative populations: Andeans increase ventilation in order to increase oxygen delivery to the foetus, while Tibetans rather increase the blood flow by production of the vasodilatatory mediator nitric oxide (NO) (Beall, 2007). Weight at birth of newborns is usually lower than at sea level for both populations, as for low-landers exposed to hypoxia (Soria et al., 2013). In conclusion, Andeans are considered to display features of classical adaptation to hypoxia and are able to cope with them for their entire life, while Tibetans show a different way of adapting with surprisingly low oxygen saturation levels. Genome-wide association studies have been performed in order to identify genetic traits responsible for such adaptation and their heritability: genes belonging to the HIF pathway, as well as genes involved in O_2 transport showed up in such studies but the functional link between specific polymorphisms and different response to hypoxia is often unclarified (Cheviron and Brumfield, 2012).

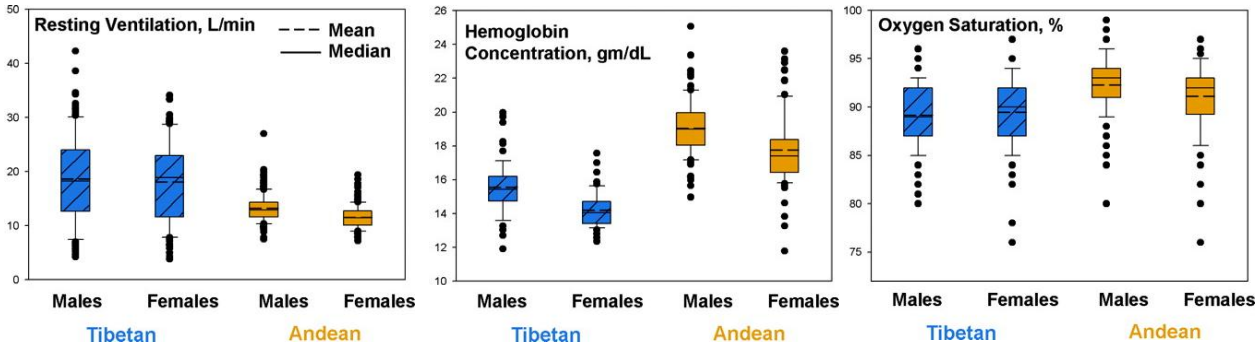

Figure 8 Resting ventilation rate, Hb concentration and oxygen saturation of the blood of Tibetan and Andean samples living at ~4000 m of altitude (adapted from (Beall, 2007)).

A few recent studies do characterize non-synonymous coding region substitution in *EGLN1*, the gene encoding for PHD2. The haplotype D4E/C127S was markedly enriched in the Tibetan population analysed compared to low-landers and the resulting mutant PHD2 protein displayed reduced interaction with p23, a heat shock protein (HPS) 90 co-chaperone that facilitates HIF-α hydroxylation by PHD2 (Song et al., 2014). The result is decreased PHD2 protein activity and increased HIF pathway activation, somehow counterintuitive if considered that Tibetans display

rather low values of Hb and Hct. Nevertheless, certain genetic traits in the *HIF2A* locus are also enriched in the Tibetan population and may account for compensatory mechanisms (Bigham and Lee, 2014). Increased HIF-1α activation could even be beneficial at high altitude, since this subunit is responsible for increased respiration and nitric oxide production (Melillo et al., 1995; Kline et al., 2002). On the other hand, Lorenzo et al. showed that the same missense mutations increase PHD2-mediated de-stabilization of HIF-2α in hypoxic Hep3B cells and that the mutant protein displays lower affinity constant (K_m) value for O_2. Moreover, proliferation of the erythropoietic progenitors derived from Tibetans carrying the D4E/C127S haplotype was impaired under hypoxic culture conditions (Lorenzo et al., 2014). Collectively, these data suggest a complicated and still unclear relation between the genetic traits found to be associated to high altitude population and the phenotypic consequence on systemic adaptation to hypoxia.

1.2.2 Cellular response to hypoxia: the pVHL/PHD/HIF pathway

The response of nucleated mammalian cells to hypoxia is mediated by the stabilization of the ubiquitously expressed transcription factor HIF, which activates a number of genes involved in anaerobic glycolysis, angiogenesis, erythropoiesis, iron uptake and cell proliferation control. The HIF pathway has a central role in the systemic response described above, mainly in adaptation to chronic hypoxia rather than in the acute phase. Heterozygous mutant Hif1a mice show in fact normal acute ventilatory response but impaired chronic ventilatory adaptation (Kline et al., 2002).

Hypoxia-inducible factors were discovered in the early 90's, when Semenza and co-workers isolated a factor binding the downstream enhancer of the *EPO* gene in hypoxic nuclei of Hep3B cells (Semenza and Wang, 1992). Later on, characterization of the purified factor demonstrated the heterodimeric nature of the HIF complexes, which consist of a hypoxia-inducible, ~120 kDa α subunit and a constitutive 91-94 kDa β subunit (Wang and Semenza, 1995). Other groups managed to isolate the same factors in different cell lines exposed to hypoxia (Maxwell et al., 1993). Three HIF-α subunits have been identified so far, encoded by different genes and conserved in *Metazoa*, but absent in bacteria, yeast and plants (Taabazuing et al., 2014). The basic structure of HIF-1α, -2α and -3α proteins is similar and is depicted in figure 9. A basic helix-loop-helix (bHLH) domain is present at the N-terminus and capable of DNA binding, 2 Per-Arnt-Sim homology (PAS) domains are responsible for protein-protein interaction with the β subunit, the oxygen-dependent degradation domain (ODDD) is involved in HIF stability regulation and the N- and C-terminus activation domains (NAD and CAD) confer transcriptional activity. The inhibitory domain (ID) is required for normoxic repression of the CAD (Lisy and Peet, 2008). HIF-1α and -2α can form transcriptionally active dimers with HIFβ or ARNT (aryl hydrocarbon receptor nuclear

translocator), while HIF-3α shows no or weak transcriptional activity and is considered as a negative regulator of the other two isoforms. The human *HIF3A* locus is subjected to alternative splicing and can originate different variants. Among these are a hypoxia-induced isoform, termed inhibitory PAS domain protein (IPAS), capable of dimerizing with HIF-1β and inhibit transcription of target genes, and a transcriptionally weak isoform, termed neonatal and embryonic PAS (NEPAS), also able to form heterodimers with HIF-1β (Makino et al., 2001; Yamashita et al., 2008). Therefore, HIF-3α could play a role in modulating the hypoxic response mediated by HIF-1α and -2α. Importantly, HIF-β displays a similar structure but lacks the ODDD and is thus not regulated according to oxygen tension (Semenza et al., 1997).

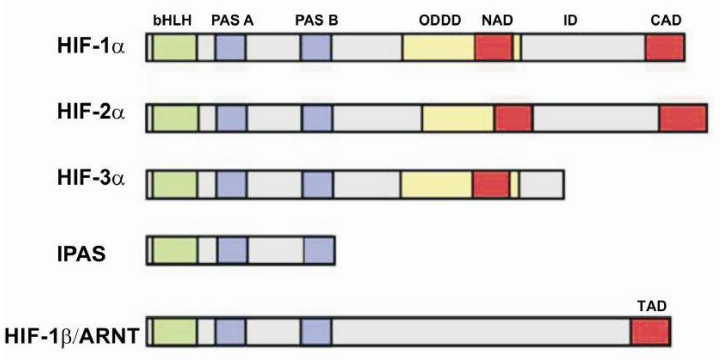

Figure 9 Structural domains of HIF-α, IPAS and HIF-β monomers (Lisy and Peet, 2008).

The ODDD is crucial for the oxygen-dependent regulation of HIFs, which occurs at the protein stability level rather than at the transcriptional level, allowing a very fast response in case of hypoxia. The ODDD contains two proline residues (Pro402 and Pro564 for human HIF-1α, Pro405 and Pro531 for human HIF-2α) that are hydroxylated in the presence of O_2 by the PHD enzymes. PHD-1, -2 and -3 belong to the superfamily of non-heme iron/2-oxoglutarate-dependent hydroxylases (Bruick and McKnight, 2001; Epstein et al., 2001). In normoxic conditions, PHDs catalyse a reaction in which O_2 and 2-oxoglutarate are converted to CO_2 and succinate, with the concomitant hydroxylation of the proline residues in the HIF-α subunits (Kallio et al., 1999). Hydroxylated HIF-α is recognized by an E3 ubiquitin-protein ligase, pVHL, and targeted for proteasomal degradation (Ohh et al., 2000). Of note, oxygen availability represents the limiting factor for the reaction, making PHDs the actual oxygen sensors of mammalian cells. Also, due to their high O_2 Michaelis constant (K_m) value, and thus low O_2 affinity, PHDs have optimal oxygen-sensing properties under all physiologically relevant oxygen concentrations (Stiehl et al., 2006). In hypoxia, the hydroxylation reaction cannot take place and HIF-α subunits are stabilized, translocate into the nucleus and dimerize with HIF-β, forming the transcriptionally active complex (Schofield and Ratcliffe, 2004). The heterodimer recruits co-factors such as p300/CBP in order to

regulate transcription of target genes (Arany et al., 1996). Another member of the hydroxylase enzyme family is the factor inhibiting HIF (FIH), which hydroxylates an asparagine residue within the CAD (Asn803 in human HIF-1α and Asn851 in human HIF-2α), blocking the interaction between HIF-α and p300 (Lando et al., 2002). Figure 10 summarizes the oxygen-dependent regulation of the HIF pathway: PHD-2 and -3 are HIF target genes themselves and their expression is increased in hypoxia, providing an intrinsic feedback mechanism (Ang et al., 2002; McDonough et al., 2006).

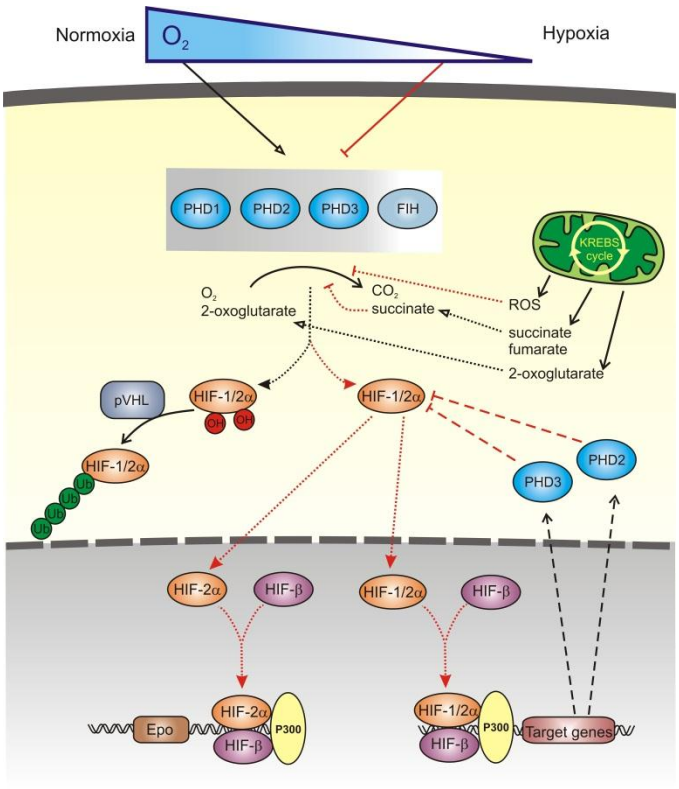

Figure 10 Simplified overview of the pVHL/PHD/HIF pathway, its regulation by oxygen tension and cross-talk with mitochondria metabolism (adapted from (Wenger and Hoogewijs, 2010)).

While HIF-1α expression can be found in all the nucleated cells of metazoan species, HIF-2α expression seems to be restricted to specific cell types, such as renal interstitial cells, hepatocytes, epithelial cells of the duodenum, cardiomyocytes and astrocytes in rodents exposed to systemic hypoxia (Patel and Simon, 2008). Global knockout for both Hif1a and Epas1 (the genes encoding for HIF-1α and HIF-2α, respectively) showed lethal phenotype during embryonic life, suggesting a fundamental role of both factors in development. Hif1a$^{-/-}$ mice died at E11 due to cardiovascular malformation and open neural tube defects (Iyer et al., 1998; Kotch et al., 1999). Different phenotypes have been reported for global Epas1 knockout: bradycardia due to insufficient

catecholamine production (Tian et al., 1998), impaired remodelling of the primary vascular network (Peng et al., 2000), multiple organ pathology, metabolic abnormalities and impaired homeostasis of ROS (Scortegagna et al., 2003) and anaemia with impaired Epo production in one particular viable strain (see below) (Scortegagna et al., 2005). $Arnt^{-/-}$ mice showed aberrant placental architecture and vasculature (Adelman et al., 2000), while knockdown and transgenic models for Hif3a revealed a role for this isoform in heart and lung development (Yamashita et al., 2008; Huang et al., 2013).

How do HIF complexes exert their function as master regulators of the hypoxic response? As transcription factors, they bind to the so-called hypoxia-response element (HRE), present in the promoters and regulatory regions of hypoxia-regulated genes. Analysis of validated HIF target genes has identified the sequence 5′-RCGTG-3′ (R = A or G) as the core HIF-binding site (HBS) (Wenger et al., 2005). In many HREs, the HBS is followed after 0-8 nucleotides by the sequence 5'-CACA-3', the "CACA repeat" (Semenza, 2013). Single mutagenesis of the HBS or of the CACA sequence within the 3' *EPO* HRE (see below) abrogated hypoxic induction of the gene, indicating that the two elements are equally important for the functionality of the HRE (Semenza and Wang, 1992). To date, no factor binding to the CACA repeat has been identified. Recent studies have made use of pan-genomic techniques, such as chromatin immunoprecipitation-sequencing (ChIP-seq), in order to screen the entire genome for HBS (Xia and Kung, 2009; Tanimoto et al., 2010; Schödel et al., 2011; Mimura et al., 2012; Schödel et al., 2012). The number of identified HBS is in the order of 500 for any particular cell line. Even though the results vary according to the stringency of the HBS/HRE definition, it is interesting to note that for example HIF-1α binding sites are usually closer to the transcriptional start site of target genes compared to HIF-2α binding sites, which often lie at promoter-distant regions (possibly enhancers). Additionally, the distribution of HBS in the genome seems to be cell-line specific, with few overlap between the cell lines tested (Schödel et al., 2013). Different approaches, such as genomic-wide transcript analysis in HIFs knockout/knockdown cell lines, lead to the identification of many more genes (in the order or hundreds or even thousands) that are directly or indirectly regulated by hypoxia (Elvidge et al., 2006; Warnecke et al., 2008; Xia et al., 2009). Again, no marked overlap can be seen between the tested cell lines. HIF-1α or -2α specificity does not depend on the DNA sequence of the binding site: no obvious difference in the core HRE composition could be observed. Isoform specificity rather lies in recruitment of different co-factors and in tissue-specific expression pattern (Wenger et al., 2005). A recent report in literature suggested that the transcription factors STAT3C (signal transducer and activator of transcription 3) and USF2 (upstream stimulatory factor 2) are recruited *in vitro* on promoters/enhancers of HIF-1α and -2α target genes, respectively, thus playing a role in HIF isoform specificity (Pawlus et al., 2013).

Examples of genes regulated in hypoxia include glycolytic enzymes, such as phosphoglycerate kinase 1 (PGK-1) and lactate dehydrogenase A (LDHA), that promote the switch form aerobic metabolism to anaerobic glycolysis, in order to produce energy in form of ATP molecules even in the absence of O_2. The constitutive activation of anaerobic metabolism, which is remarkably less efficient than the aerobic one, is known as "Warburg effect" and was first described as a mechanism used by tumour cells to survive the hypoxic micro-environment typical of cancers. Genes involved in oxygen transport and vascular remodelling are also HIF targets: Epo, transferrin (TF), vascular endothelial growth factor (VEGF) and nitric oxide synthases (NOS) are the most well-known examples. The list of validated HIF-target genes is long and include up- and down-regulated pathways that will ultimately promote adaptation of the cells to the stress conditions of reduced O_2 availability (Wenger et al., 2005; Semenza, 2013). Recent reports revealed the HIF-dependent regulation of long non-coding RNAs as well, another level of the complex adaptation of mammalian cells to hypoxia (Camps et al., 2014; Choudhry et al., 2014). Moreover, one report identifies a strikingly new effect of the transcriptional response to hypoxia: the release of pre-bound promoter-paused RNA polymerase 2 by binding of HIFs (Choudhry et al., 2014).

1.3 Transcriptional regulation of the *EPO* gene in hypoxia

1.3.1 Regulatory regions in the *EPO* locus

Since the discovery of Hep3B and HepG2 as Epo-expressing *in vitro* models and the kidney as the major source of Epo *in vivo*, it was clear that *EPO* gene is regulated by hypoxia (Goldberg et al., 1987), by hypoxia-mimetics such as $CoCl_2$ (Beru et al., 1986) and by anaemia (Semenza et al., 1990). This fact was further confirmed by the discovery of HIF-1 as a heterodimer binding to the 3' enhancer of the *EPO* gene (Semenza et al., 1991). Comparison between the mouse Epo and human *EPO* genes and surrounding sequences revealed homology not only of the coding part, but also of the flanking regions, suggesting an important role for such regions in the regulation of the gene. In particular, the fragment directly upstream of the transcriptional start site (TSS), the first exon, the first intron and the fragment directly downstream of the poly-adenylation site (polyA) are the most conserved (Galson et al., 1993). *EPO* is present in single copy on the long arm of chromosome 7 in human and on chromosome 5 in mouse and consists of 5 exons and 4 introns. Most of the knowledge we have concerning Epo hypoxic regulation by *cis*-acting sequences comes from *in vitro* experiments in hepatoma cell lines and from *in vivo* studies with transgenic mice carrying different fragments of the *EPO* genomic locus. Two peculiarity of Epo regulation are worth

to be mentioned: it occurs at the transcriptional level only (and not at the RNA or protein stability levels (Gordeuk et al., 2005)) and it is restricted to very specific Epo-producing cell types. The molecular basis of Epo tight tissue specificity remains largely unknown.

Figure 11A summarizes the constructs that have been used to characterize Epo regulatory regions. The first transgenic mouse was generated with a 4 kb fragment containing the human *EPO* gene, 0.4 kb upstream of the TSS (encompassing the promoter) and 0.7 kb downstream of the polyA. These animals were profoundly polycythemic, due to promiscuous expression of Epo and elevated Epo serum levels. Nevertheless, increased Epo mRNA could be detected in liver, but not kidney, of anaemic mice, indicating the presence of a liver-inducible element (LIE) within this fragment (Semenza et al., 1989). Expansion of the region upstream to the TSS to 6 kb decreased Epo ectopic expression, suggesting a sequence between -0.4 and -6 kb is responsible for a general repressive effect on *EPO* transcription (the so-called negative regulatory element or NRE). Hypoxia inducibility was maintained in the liver and absent in the kidney (Semenza et al., 1990). The same authors further increased the size of the 5' flanking sequence to 14 kb and could show hypoxia inducibility also in the kidney of transgenic mice exposed to bleeding anaemia. They concluded that the kidney-inducible element (KIE) must be located between -6 and -14 kb from the TSS in the human *EPO* locus (Semenza et al., 1991). Some of the transgenic lines generated with these constructs still showed a polycythemic phenotype which was due to dramatically increased Epo levels in the liver. Normal Epo levels and anaemia-induced transgene expression in both liver and kidney were obtained by Madan et al. by increasing the size of the 3' flanking region to 8.5 kb from the polyA, speaking for the presence of a negative regulatory liver element (NRLE) between 0.7 and 8.5 kb downstream of *EPO* (Madan et al., 1995). To test whether the intron sequences are important for *EPO* regulation, a construct containing 0.4 kb of the upstream region, 0.7 kb of the downstream region and a minigene lacking introns II-IV was generated: Epo ectopic expression pattern with liver, but not kidney, inducibility was observed, similar to the constructs containing all the introns (Madan et al., 1995). Later studies also confirmed the importance of intron I, but not of introns II-IV, in hypoxic *EPO* regulation (Obara et al., 2008). Again, some of the transgenic mouse strains displayed high Hct values, which correlated with the copy number of the construct inserted into the genome. The presence of open chromatin structures within the putative KIE and LIE, and thus their potential role as regulatory regions, was proven by DNaseI hypersensitivity site (HS) mapping in nuclei of kidney and liver of anaemic mice (Semenza et al., 1991; Köchling et al., 1998).

The next "generation" of vectors used to study Epo regulation consists of reporter genes under the control of the mouse Epo locus (figure 11B). LacZ was first used to confirm the presence of a liver-

specific silencing element within 1.2 kb from the polyA of the Epo gene (Haidar et al., 1996). The most important Epo reporter model was generated by inserting the GFP gene into a BAC clone of 180 kb: 60 kb upstream and 120 kb downstream of the gene. This construct fully recapitulated Epo expression pattern/hypoxic inducibility and was used to characterize and isolate REPCs as well (see figure 5) (Obara et al., 2008; Pan et al., 2011). Moreover, mutated versions of this model were useful to assess the functionality of different transcription factor binding sites, such as the GATAs and HIF itself (see below) (Suzuki et al., 2011). The newest construct generated by the group of Yamamoto encompasses 22 kb 5' to the TSS and 163 kb 3' to the polyA: once again, GFP expression pattern follows the physiological one for Epo. This BAC clone was used to study embryonic Epo expression in neural crest cells (Suzuki et al., 2013).

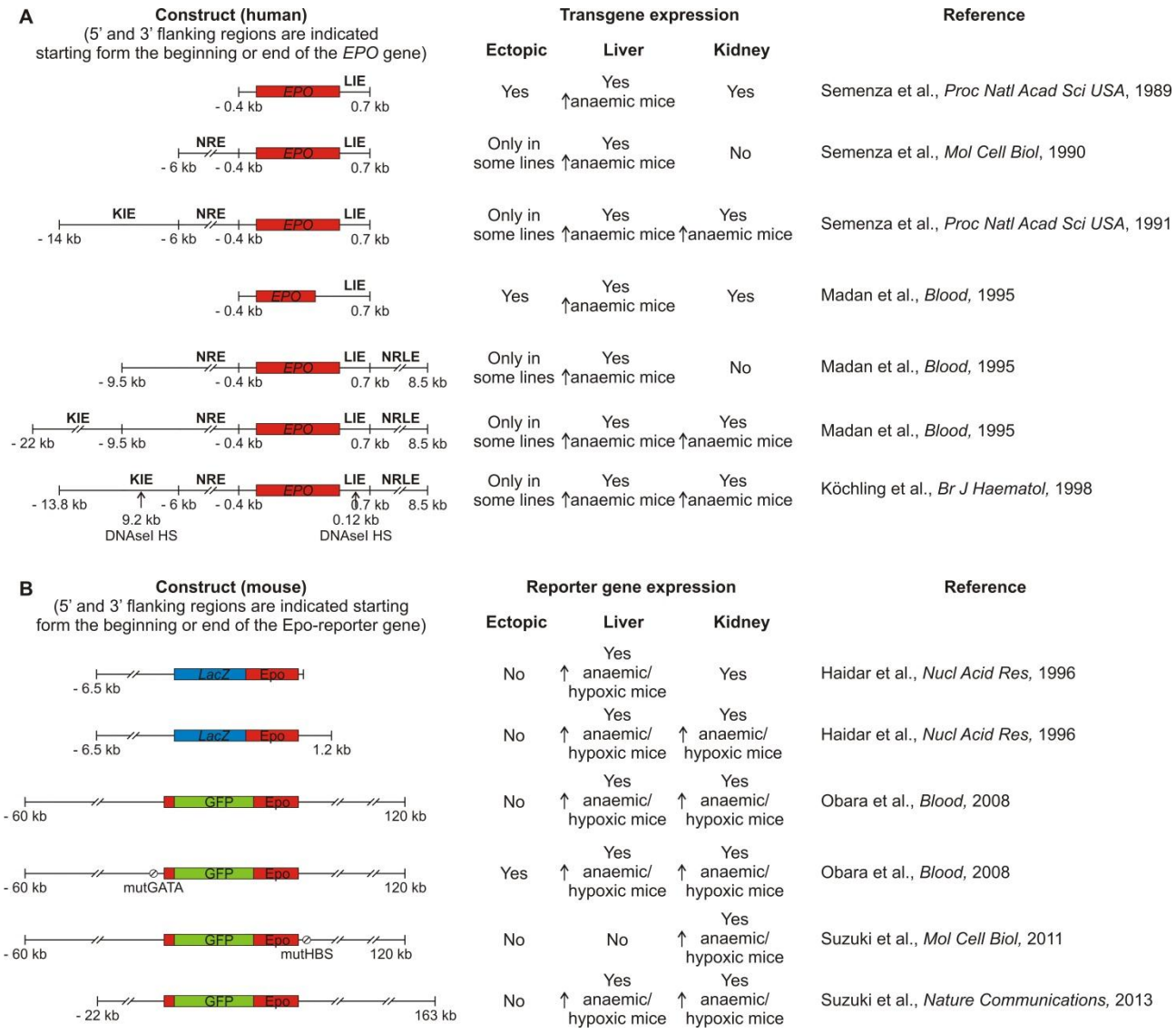

Figure 11 Schematic representation of the human *EPO* locus (**A**) and reporter mouse Epo locus (**B**) constructs used to generate trangenic mice. Transgene expression pattern and references are indicated.

The different, tissue-specific regulatory regions identified with transgenic animals have been extensively characterized with the help of *in vitro* models, starting from the promoter. Reporter assays in Hep3B led to the identification of the Epo minimal promoter (EpoProm), a sequence of 117 bp upstream of the TSS with weak activity (Gupta and Goldwasser, 1996). Moreover, binding of the murine factor HAF (hypoxia-associated factor, homologous of the human squamous cell carcinoma antigen recognized by T cells (SART)-1) has been found in EpoProm. HAF mRNA is ubiquitous in mice and highly abundant in foetal liver, while its expression declines in adult liver, mirroring Epo hepatic regulation. HAF knockdown resulted in reduced, but not absent, Epo hypoxic

induction in Hep3B cells (Gupta et al., 2000). The EpoProm also contains a putative HRE at -180 bp position, but reporter assays demonstrated the lack of functionality of this sequence (Blanchard et al., 1992). Other important factors binding in the promoter are the GATA factors 2, 3 and 4. GATAs are important regulators of haematopoiesis (Shimizu and Yamamoto, 2005) and GATA binding sites have been identified in the EpoProm (Imagawa et al., 1997). GATA-2 and -3 seem to have an inhibitory effect on *EPO* transcription, while GATA-4 has the opposite effect, as demonstrated by knockdown and overexpression experiments in hepatoma cell lines. ChIP assays confirmed the binding of GATAs to EpoProm *in vitro* and *in vivo*. Given the increasing expression of GATA-2 and -3 and decreasing expression of GATA-4 in the developing liver, these factors have been hypothesised to play a role in silencing Epo expression in the adult liver (Dame et al., 2004). Additionally, the importance of the GATA binding site was proven by its mutation in GFP reporter mice: in the mutated strain, ectopic GFP expression was observed, confirming the inhibitory effect of this element on *EPO* transcription (Obara et al., 2008). The Wilms tumour suppressor (Wt-1) is an additional transcription factor that has been shown to bind and activate EpoProm *in vitro*. Since its expression pattern resembles the one of Epo, Wt-1 could be important in maintaining Epo tissue specificity (Dame et al., 2006).

Due to the availability of liver-derived cellular models, the LIE (or 3' Epo enhancer) has been studied in depth over the past few years. An HRE consensus sequence is present within the LIE, at ~120 bp from the end of the polyA, and consists in the typical HBS sequence followed after 7 bp by a CACA repeat (Pugh et al., 1991; Semenza and Wang, 1992). Reporter assays in human cell lines showed that this HRE is responsible for hypoxic induction of Epo mRNA by synergizing with the minimal EpoProm (Beck et al., 1991; Imagawa et al., 1991; Blanchard et al., 1992). Mutation of the HBS in reporter mice abrogated hypoxia-inducible Epo expression in the liver, but not in the kidney, confirming the liver-specificity of this element and suggesting the presence of an additional HRE regulating renal Epo expression (Suzuki et al., 2011). As mentioned above, despite no factor binding to the CACA repeat has been identified so far, this element is necessary, but not sufficient, for Epo induction by hypoxia (Semenza and Wang, 1992). A DR-2 site (direct repeat of two steroid hormone receptor half sites, separated by 2 bp) is present in the 3' enhancer and has been shown to bind non-oxygen regulated protein complexes. Mutation of the DR-2 markedly inhibited Epo expression in hypoxia (Blanchard et al., 1992). Different transcription factors, belonging to the steroid hormone receptor family, can bind to the DR-2 and hepatic nuclear factor-4α (HNF-4α) has been identified as one of them *in vitro* (Galson et al., 1995). HNF-4α is expressed in hepatocytes and renal cortex and is replaced on the DR-2 site by the retinoic acid receptor (RXRα) in embryonic mouse liver, suggesting the possibility that different members of the steroid hormone

receptor family can bind the DR-2 repeat in the LIE (Makita et al., 2001). As for the other HIF-target genes, the general activator of transcription p300/CBP is recruited upon hypoxia to the 3' enhancer of *EPO* (Arany et al., 1996). Besides the oxygen-regulated HIF complex, another, constitutive transcription factor complex has been identified bound to the LIE: proteins such as activating transcription factor 1 (ATF-1) and cAMP responsive element binding protein (CREB) are part of the latter (Semenza and Wang, 1992). The final model for *EPO* transcriptional regulation is depicted in figure 12. In hypoxia, the HRE is bound by the stabilized HIF-2 complex, HNF-4α interacts with the HIF-α subunit and both HIF-α and -β interact with p300/CBP, forming an active complex that enhances *EPO* transcription up to 100 folds. Only one report in literature showed the presence of a putative HRE sequence at -9.2 kb from the TSS, within the KIE. This sequence is homologous to the 3' enhancer HRE and is located in a DNaseI HS in nuclei of kidney of anaemic mice. No functional characterization of this sequence has been reported so far (Köchling et al., 1998).

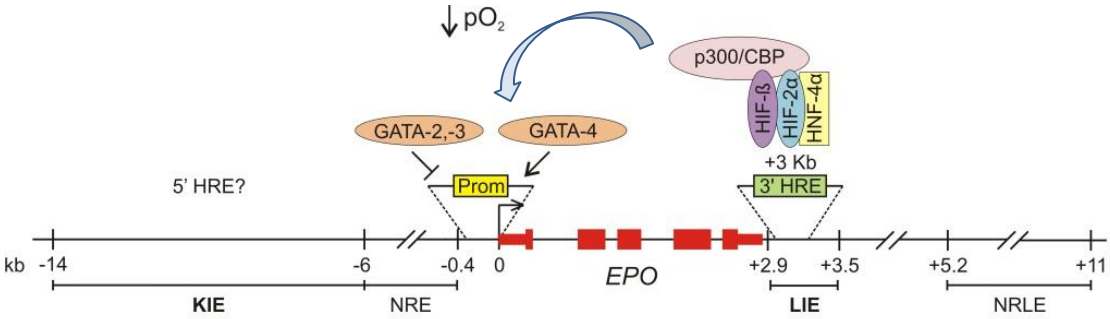

Figure 12 Scheme depicting the human *EPO* genomic locus, with the most important regulatory DNA sequences and transcription factors. The presence of an HRE within the KIE (5' HRE) is still debated. Numbers indicate the distance in kb from the TSS (-: upstream, +: downstream).

Epigenetic regulation participates in Epo expression in the form of CpG methylation. DNA methylation is known to occur on position 5 of cytosine in the CG dinucleotide and to inhibit transcription via recruitment of chromatin-remodelling complexes that keep the DNA locus close (Alberts et al., 2002). *EPO* promoter and 5' untranslated region (5'UTR) are indeed located in a CpG island and methylation of this region inversely correlates with Epo expression *in vitro* (Yin and Blanchard, 2000). Moreover, methylation of the HBS in the 3' enhancer seems to prevent HIF binding to the DNA and also inversely correlates with Epo expression in cell lines and tissues (Wenger et al., 1998; Stolze et al., 2002). These reports show the requirement of a methylation-free HBS in order to have HIF binding to the 3' enhancer and Epo induction in hypoxic conditions. Interestingly, treatment of different cancer cell lines with the DNA methylation inhibitor 5-aza-2′-deoxycytidine rescued Epo hypoxic expression in neuroblastoma cells but had no effect on Hep3B

cells, where the *EPO* locus is already de-methylated and open to transcription (Steinmann et al., 2001). DNA methylation can thus be an important factor contributing to *EPO* gene silencing in tissues other than kidney and liver.

1.3.2 HIF/PHD isoforms regulating Epo

EPO is often used as a paradigm for hypoxia-induced gene transcription and is indeed regulated by the canonical pVHL/PHD/HIF pathway. *VHL* is known to be a tumour suppressor gene and a number of mutations can cause familial erythrocytosis as well, supporting its role as an important regulator of Epo (Bader and Hsu, 2012). Which PHD/HIF isoforms specifically control Epo expression? The answer to this question comes from *in vitro* knockdown experiments, conditional knockout mice generation and genetic diseases where mutations in the PHD/HIF genes lead to impaired Epo expression. Among the genes encoding for PHD-1, -2 and -3 (Egl9 homolog (*EGLN*)-*2*, *-1* and *-3*, respectively), inducible knockout models for PHD-2 showed increased Epo levels, polycythemia and angiogenesis, accompanied by cardiomyopathy and premature death (Minamishima et al., 2008; Takeda et al., 2008). Consistently, global knockout for PHD-2, but not PHD-1 or -3, resulted in embryonic lethality at E12-14 due to placental and cardiac defects (Takeda et al., 2006). Additional evidence for a major role of PHD-2 in regulating Epo expression is given by the identification of mutations in *EGLN1*, but not *EGLN2* or *3*, in families affected by congenital erythrocytosis (Percy et al., 2006; Percy et al., 2007; Ladroue et al., 2008). The first proof of Epo as a HIF-2α-specific target gene came from *in situ* hybridization studies in ischemic rat kidneys showing expression of HIF-2α in peritubular fibroblasts, the putative REPCs (Rosenberger et al., 2002). *In vitro* confirmation of HIF-2α prominent role was given by Warnecke at al., who made use of the RNA interference (RNAi) approach to knockdown the two isoforms: only HIF-2α knockdown significantly decreased Epo hypoxic induction (Warnecke et al., 2004). As mentioned above, one mouse model of global Epas1 knockout turned out to be viable and showed decreased renal Epo levels and anaemia (Scortegagna et al., 2005). Liver-specific VHL knockout resulted in polycythemia in mice and this phenotype could be rescued by concomitant deletion of Arnt or Epas1 but not Hif1a (Bigham et al., 2010). Furthermore, experiments with hepatic and renal single $Epas1^{-/-}$ mice confirmed the role of HIF-2α, rather than HIF-1α, in regulating Epo expression in both organs (Rankin et al., 2007; Kapitsinou et al., 2010). In order to avoid embryonic lethality, acute ablation of Epas1 after birth was performed and led to anaemia in adult mice (McMullin, 2009). Evidences for HIF-2 specificity of *EPO* regulation in humans are derived from mutations identified in *EPAS1* causing hereditary erythrocytosis (a few of the most recent examples: (McMullin et al., 2005; Clodagh et al., 2013; Bento et al., 2014; Moulard et al., 2014)), whereas no mutations in *HIF1A* or *HIF3A* have been reported so far. In order to produce functional RBCs, iron

uptake and release from the body storage is increased during erythropoiesis: iron metabolism is indeed linked to oxygen-sensing via HIF-2α (Mastrogiannaki et al., 2009). DMT-1 (divalent metal transporter 1) and DcytB (duodenal cytochrome *b*) are examples of HIF-2-dependent genes involved in iron uptake (Haase, 2010) and the 5'UTR of HIF-2α mRNA itself contains iron-responsive elements (Zimmer et al., 2010). The cross-talk between iron metabolism and HIF-2 additionally supports the primary role of HIF-2α in Epo regulation. Moreover, genome-wide association studies performed comparing lowlanders to highlanders identified polymorphisms in *EGLN1* and *EPAS1* in association with RBC mass, confirming that these two genes are indeed involved in Epo expression control (Bigham et al., 2010; van Patot and Gassmann, 2010). Taken together, these data point in the direction of pVHL/PHD-2/HIF-2α as the main axis regulating Epo expression in hypoxia. It must be mentioned that experiments performed both *in vitro* and *in vivo* demonstrated binding of HIF-1α and HIF-2α on Epo 3' HRE, suggesting the possibility of binding (but not functionality) of both isoforms or of different HIF-α-specificity according to the tissue/cell line tested (Stolze et al., 2002; Yeo et al., 2008). A model targeting REPCs *in vivo* is still missing and would be required to dissect the role of different PHD/HIF isoforms at the physiological site of Epo production.

1.3.3 Epo negative feedback regulation

As shown in figure 2C, Epo plasma concentration decreases during chronic hypoxia before the Hct values actually reach normal levels and restore the O_2-carrying capacity of the blood (Jelkmann, 1982). This drop is due to a decrease in renal Epo mRNA and protein abundance and is independent of changes in extracellular Epo levels (Rosa et al., 1978; Gordeuk et al., 2005). Therefore, an intrinsic feedback loop in the pVHL/PHD/HIF pathway in REPCs has been proposed, but not yet proven, as the underlying mechanism. PHD-2 and -3 are indeed HIF-target genes and could be involved in decreasing Epo expression after a certain time of hypoxic exposure (Ang et al., 2002; McDonough et al., 2006). Another important issue is the high degree of plasticity of the HIF pathway: even small changes in PHD/HIF levels or activity can lead to big changes in the downstream effects. If HIF-α isoform's abundance is increased, for example, the system would be saturated because PHDs are not fully active even in normoxic conditions and stabilization of HIF complexes would occur. It is then easy to imagine that regulators of PHDs/HIFs levels or activity can be involved in the feedback mechanism (Kaelin, 2007; Lee and Percy, 2011). Many peptides and proteins have been identified as PHD interactors and some of them do regulate the stability or activity of the enzymes (Furlow et al., 2009). Moreover, intermediated of the Krebs cycle, such as succinate or fumarate, reactive oxygen substances, like ascorbate, transition metals, NO and ROS can all regulate PHDs activity (see figure 10) (Wenger and Hoogewijs, 2010; Wenger and Kurtz,

2011). In conclusion, no clear mechanism has been proven so far to be responsible for the drop in Epo levels in chronic hypoxia and a kidney-derived, Epo-expressing cell model would be useful to investigate further in this direction.

1.4 Pathophysiology of Epo

Two main and opposite clinical conditions arise from impaired Epo synthesis: when Epo is overproduced, erythrocytosis or polycythemia can occur, whereas insufficient Epo production results in anaemia. Besides these two diseases, described in details below, medical procedures interfering with the main Epo-producing organ, the kidney, can result as well in impaired erythropoiesis. Kidney allotransplantation represents such a case and was used to treat anaemia before the introduction of rhEpo. In patients undergoing kidney transplantation, Epo levels usually increase within the first few days, probably due to blood loss during the operation (Eckhardt et al., 1990) and in 10% of the cases polycythemia can occur (Davis, 1987). Interestingly, not only the transplanted, but also the native kidney plays a role in increasing Epo levels after transplantation (Martino et al., 1994). This fact supports the hypothesis according to which even damaged kidneys retain their ability to produce Epo but are somehow not able to couple Epo synthesis and O_2-sensing when the organ is not functioning correctly. Kidney functionality seems indeed to be a prerequisite to physiological Epo production: when the kidney is able to exert its excretory function, oxygen-sensing and Epo synthesis appear to be normal. In particular, correct function of the proximal tubule (the main oxygen-consuming segment of the nephron) seems to be the most relevant in order to maintain the O_2 gradient and thus the oxygen-sensing capacity of the kidney (Wenger and Kurtz, 2011).

1.4.1 Erythrocytosis

Erythrocytosis is a synonymous of polycythemia and refers to inappropriately increased RBC mass. Table 2 summarizes the classification and aetiology of the disease: erythrocytosis can be classified into primary, when defects intrinsic in the haematopoietic progenitors lead to excessive RBC formation, and secondary, when impaired Epo production is the cause for increased Hb content of the blood. In addition, both primary and secondary erythrocytosis can be acquired or congenital (i.e. due to inherited mutations, also called familial). Hereditary pattern of the congenital disease can be both autosomal recessive and autosomal dominant, depending on the mutation. Diagnostic criteria are the following: Hb concentration > 18.5 g/dL in men and 16.5 g/dL in women (McMullin, 2009). An absolute or true erythrocytosis is present only when the red cell mass is

greater than 125% of that predicted for sex and body mass (Clodagh et al., 2013). A group of individuals for whom the cause of erythrocytosis is still not clear exists and is referred to as idiopathic erythrocytosis.

Primary erythrocytosis	Secondary erythrocytosis
Acquired	**Acquired**
Polycythemia vera, clonal expansion of erythroid progenitors in the bone marrow	Central hypoxia (chornic lung disease, cardiovascular diseases, high altitude) Local hypoxia (end-stage renal disease, post-renal transplant erythrocytosis) Pathological erythropoietin production (tumour-related) Iatrogenic (erythropoietin, Epo-stimulating agents, androgens)
Congenital	**Congenital**
EpoR mutations	High oxygen-affinity haemoglobins Bisphosphoglycerate mutase deficiency Oxygen-sensing pathway gene mutations (VHL, EGLN1, EPAS1)

Table 2: classification and aetiology of erythrocytosis (adapted from (Gordeuk et al., 2005; McMullin et al., 2005)).

The most common form of the disease, with around 1.6 cases/100`000/year in Europe, is called *polycythemia vera* and represents a primary, acquired condition usually arising from expansion of mutated clones within the bone marrow (Moulard et al., 2014). Primary congenital erythrocytosis is rarer and most of the cases are caused by mutations in EpoR resulting in constitutive activation of the pathway and subsequently in inappropriate proliferation of RBC precursors (Bento et al., 2014).

Any condition that increases Epo production causes instead acquired secondary erythrocytosis. The most common examples include pathological Epo synthesis by kidney, liver and brain tumours, iatrogenic stimulation (due to Epo-stimulating drugs), central and local hypoxia of different aetiology, such as cardiovascular diseases, exposure to high altitude, chronic lung disease, carbon monoxide poisoning and kidney transplantation (Clodagh et al., 2013). Secondary congenital erythrocytosis is due to inherited mutations in genes involved in oxygen

sensing and transport. Increased O_2 affinity of mutated Hb causes polycythemia because it prevents oxygen dissociation in peripheral tissues, leading to tissue hypoxia and compensatory erythropoiesis (Percy et al., 2009). As mentioned above, 2,3-DPG is one of the most important regulator of Hb affinity and loss-of-function mutations in the enzyme responsible for 2,3-DPG synthesis, bisphosphoglycerate mutase, have been reported in families affected by erythrocytosis (Rosa et al., 1978; Hoyer et al., 2004). It is not surprising that mutations in genes encoding for the pVHL/PHD2/HIF-2α axis have a great impact on *EPO* transcriptional control and a number of mutations have been indeed associated with secondary familial erythrocytosis (Bento et al., 2014). From a molecular point of view, these naturally occurring mutations are extremely useful to study the conformational change and impact on functionality of the mutated proteins; a few examples are given in the next paragraph. It must be mentioned that not all the mutations identified result in increased Epo levels: some patients with gain-of-function mutations clearly leading to increased activity of the HIF pathway display Epo concentrations within the normal range but increased Hct, suggesting additional primary changes in haematopoietic precursors (Perrotta et al., 2013).

The main symptom experienced by polycythemic patients is increased viscosity of the blood due to high RBC mass, which can cause fatigue, chest and abdominal pain, myalgia, headache and blurred vision. The most clinically relevant consequence is definitely the increased risk of thrombosis (formation of blood clot and possibly vessel occlusion). Therapy is different according to the patient's symptoms and the degree of erythrocytosis but it usually involves venesection (or phlebotomy, surgical opening of a vein to remove excessive RBC mass) and administration of anti-coagulant drugs, such as aspirin. Novel therapies in clinical trials for congenital erythrocytosis aim to target the specific altered pathway (Clodagh et al., 2013).

Mutations in the VHL/ENGL1/EPAS1 axis associated with secondary congenital erythrocytosis

Homozygous R200W mutation in *VHL* is known to cause an autosomal recessive form of poylcythemia, initially detected in the Russian region of Chuvash (thus termed "Chuvash polycythemia") (Ang et al., 2002). This mutation results in impaired binding of pVHL to hydroxylated HIF-α subunits and increased activation of the oxygen-sensing pathway. Epo and other HIF-target gene levels are in fact elevated but, surprisingly, no malignancy is associated with Chuvash patients as for other *VHL* mutations causing multiple cancer syndromes or VHL disease (Haddad et al., 2013). Moreover, increased sensitivity of erythroid progenitors to Epo has also been demonstrated in these patients, suggesting that Chuvash polycythemia shares features of both primary and secondary erythrocytosis (Gordeuk et al., 2005). *VHL* is considered

a tumour suppressor gene and its loss-of-function is detected in > 90% of clear cell renal cell carcinomas (ccRCC), the most frequent from of kidney cancer (Frew and Moch, 2015). It is remarkable that only a few mutations in *VHL* gene have been causally associated to both erythrocytosis and VHL disease (two examples are Y157C and L188V) (Lorenzo et al., 2013). A very recent report further explores the genotype-phenotype correlations of a unique family carrying heterozygous R200W mutation in combination with an additional mutation in the same gene, R161Q. In this case, heterozygosis of the R200W mutation was not sufficient to cause erythrocytosis, as the mutant protein displayed similar but not equal activity in de-stabilizing HIF-2α as the wt protein. Interestingly, an additive effect of R200W and R161Q leads to high risk of developing kidney tumour in this family (Couvé et al., 2014). Taken together, these evidences suggest that differences in the mutated copy number carried by the patient and impact on mutant protein activity strongly influence the correlated phenotype and that a gradient of pVHL dysfunction in oxygen sensing seem to occur.

37 patients carrying 24 different mutations in the *EGLN1* gene have been identified so far. The first PHD2 mutation associated with familial erythrocytosis was the amino acid substitution P317R (Percy et al., 2006). Crystallographic studies showed that the proline residue 317 is conserved and localized within the catalytic domain of the enzyme, in close proximity to the iron-chelating residues 313 and 315 (McDonough et al., 2006). PHD2-P317R displayed indeed diminished hydroxylation capacity compared to the wt protein *in vitro* (Percy et al., 2006). A heterozygous mouse model carrying a mutation that mimics the human P371R substitution in heterozygosis was generated (Phd2P294R/+) and showed a degree of erythrocytosis comparable to the one of animals carrying a single wt allele of Phd2 (Phd2+/-). This study indicates that the effect of P317R mutation occurs through haploinsufficiency of the wt form of the protein (Arsenault et al., 2013). Besides the P371R, a number of germline missense mutations have been identified, often within PHD2 catalytic domain (P200Q, N203K, K204E, D254H, G285R, W334R, K291I, P304L, R371H, H374R and K423E) (Bento et al., 2014). The functional consequence of these mutations on protein structure and activity is not always clear. Frame-shift and nonsense mutations also have an impact on PHD2 functionality and have been associated to congenital polycythemia (Bento et al., 2014). Among the missense mutations, H374R was also linked to recurrent paraganglioma, a neuroendocrine neoplasm that can develop at different body sites, suggesting that *EGLN1* can act as a tumour suppressor gene (Ladroue et al., 2008). Interestingly, one germline mutation in *EGLN2* gene, encoding for PHD1, was recently identified in a patients suffering of pheocromocytoma and polycythemia and led to decreased protein stability with consequent activation of the HIF pathway (Yang et al., 2014).

While the mutations identified in *EGLN1* result in loss-of-function of the protein, mutations in *EPAS1* usually translate into gain-of-function of HIF-2α and increased Epo production. In total, 22 patients have been reported to have heterozygous *EPAS1* mutations. Interestingly, the majority of them (I533V, P534L, M535T/V/I, G537W/R, D539E and F540L) are located in exon 12, in close proximity to Pro531, one of the hydroxylated residues on human HIF-2α (Lee and Percy, 2011). The effect could then be reduced recognition and hydroxylation by PHD enzymes (Furlow et al., 2009). Mouse models mimicking the human G537W mutation were generated, both in homozygosis and heterozygosis: the phenotype was dose-dependent erythrocytosis and pulmonary hypertension with a high degree of penetrance (Tan et al., 2013). Another well-characterized *HIF2A* mutation is the D539E, for which clearly impaired binding with PHD2 and pVHL was demonstrated *in vitro* (van Wijk et al., 2010). As for *EGLN1*, some of the described *EPAS1* mutations associate with pheocromocytoma, a neuroendocrine neoplasm of the adrenal gland, and paraganglioma, tumours that also show impairment of the pVHL/PHDs/HIFs pathway (Kaelin, 2007). The Pacak–Zhuang syndrome is a clinical condition characterized by multiple paragangliomas, pheocromocytoma and polycythemia and is due to gain-of-function mutations in *HIF2A* gene, leading to increased stabilization of the protein. Interestingly, these somatic mutations have been identified mainly in female patients (Zhuang et al., 2012; Pacak et al., 2013). Only one report describes the case of a male patient with Pacak–Zhuang syndrome (Toyoda et al., 2014), suggesting that sex-related differences, for instance in sexual hormone levels in the blood, could have an influence on the Epo-producing pathway (see below).

1.4.2 Anaemia

Anaemia is the most common haematological disorder and is defined as a reduction in the Hb content of the blood. Since Hb concentration varies according to age, gender and genetic background, there are no clear-cut diagnostic criteria, but Hb levels < 13 g/dL in men and < 12 g/dL in women represent the rule of thumb. Aetiology of anaemia is diverse and the disease can be divided at least in two categories: the number of produced erythrocytes is not adequate or the RBCs are sufficient in count but not functional, as in the case of sickle-cell anaemia (described above). Among the causes of anaemia are nutritional deficiency (iron and vitamin B_{12}), acute or chronic blood loss, haemoglobinopathy (due to mutations in the Hb-coding genes making oxygen transport not efficient), bone marrow failure and renal failure. The main symptom is thus insufficient oxygen delivery to the peripheral tissues and subsequent pallor, fatigue and dyspnoea (Hand, 2001).

Impaired Epo synthesis clearly leads to insufficient erythropoiesis and is the major drive of anaemia in patients with chronic kidney disease (CKD). CKD are a group of pathological conditions of different aetiology leading to progressive damage of the kidney, measured by decreasing glomerular filtration rate (GFR), and consequent renal failure. The last stage of CKD is referred to as end stage renal disease (ESRD) and patients in this condition need replacement therapy (dialysis, hemofiltration or transplantation) in order to survive. The number of affected people doubled over the past decade and the costs of replacement therapies are extremely high, making CKD a global challenge for health care. The prevalence of CKD in 2014 is estimated to be around 10% of the adult population in USA, more than 20 million people (data from the American Centers for Disease Control and Prevention). A complex mixture of genetic and environmental factors is at the basis of CKD. Hypertension, diabetes, hyperlipidaemia, age, obesity and smoking are common risk factors linking cardiovascular to renal diseases: the main function of the kidney is in fact to "clear" the blood from substances that are toxic or in excess and increasing the blood pressure or the amount of such substances can result in damage of the nephron system. A number of other pathological conditions, such as severe infections and auto-immune diseases, also cause the initial inflammatory insult in the kidney which can progress to CKD. A vicious cycle of inflammation and fibrosis begins at the glomerular or tubulo-interstitial sites and functionality of the organ is compromised. Decreased GFR and proteinuria (presence of proteins in the urine) are the direct consequences of renal failure (El Nahas and Bello, 2005). Anaemia can be considered as a complication of CKD and is thought to arise from damage to the architecture of the organ and the cellular sources of Epo. Besides the fact that Epo levels are blunted and do not rise appropriately to the degree of anaemia (Caro et al., 1979), CKD patients still retain the capacity to produce renal Epo when exposed to hypoxia (Chandra et al., 1988) or PHD inhibitors (Bernhardt et al., 2010) and the Hct-Epo feedback loop seems to be functional (Walle et al., 1987). Moreover, anaemic patients need decreased dosage of rhEpo when living at high altitude, in other words they respond better to the treatment, possibly due to increased endogenous Epo production (Brookhart et al., 2008). The role of hypoxia is still debated: according to the "chronic hypoxia hypothesis", progressive renal injury leads to decreased blood flow and, together with anaemia, to tubulo-interstitial hypoxia, which further supports fibrosis and kidney damage. Reports in literature showed that renal hypoxia indeed precede and can be a cause of tissue fibrosis (Fine and Norman, 2008). On the other hand, the kidney is less functional, thus oxygen consumption is decreased and REPCs would experience higher O_2 tension which does not allow them to reach the hypoxic threshold necessary for Epo production. As mentioned above, probably intact organ structure and function are needed to keep the oxygen gradient along the cortico-medullary axis and thus for REPCs to sense decreased O_2-carrying capacity of the blood and synthetize Epo. Even though hepatic Epo expression can be

increased in CKD animal models (Tan et al., 1991; Kapitsinou et al., 2010), this is not enough to normalize Hct levels and the situation in humans is still unclear. The inflammatory component of CKD certainly plays a role in decreased Epo levels: inflammatory cytokines are known to inhibit Epo production (see below) and a recent report showed that, in a CKD mouse model, REPCs can undergo trans-differentiation into myofibroblasts losing the ability to synthetize Epo (Asada et al., 2011).

The most effective treatment for anaemic patients is represented by administration of rhEpo, with approximately 95% of treated patients responding to the therapy. Anaemia arising from renal failure, chronic inflammatory disease and cancer is usually treated with parenteral injection of rhEpo and its glycosylation-modified derivates (Bunn, 2013). Recombinant Epo replaced blood transfusion therapy in the early 90's with significant increase in efficacy and safety of the treatment, as shown in figure 13.

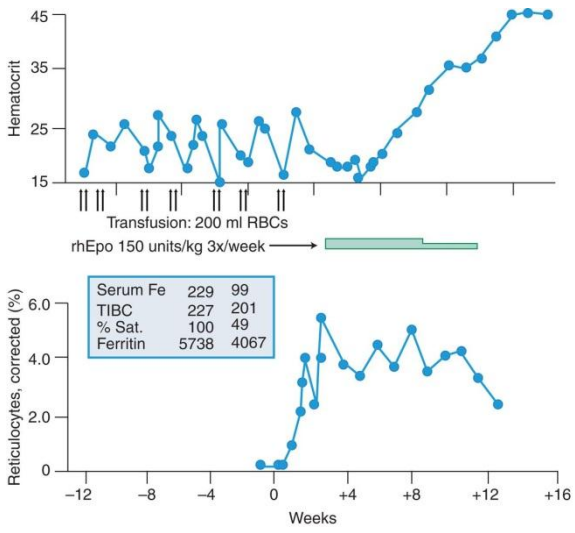

Figure 13 Response of a patient with renal failure and under dialysis to blood transfusions and rhEpo treatment. The second treatment regimen led to sustained increase in Hct and reticulocyte count. The dose of rhEpo was lowered to prevent the Hct from rising too high. The blue box shows values of serum iron, total iron binding capacity (TIBC), saturation and ferritin. Note that the severe iron overload is resolved with rhEpo treatment, indicating functional erythropoiesis has occurred (Eschbach et al., 1987).

Possible side effects of rhEpo therapy are thrombosis, due to excessive RBC number (Singh et al., 2006; Phrommintikul et al., 2007), or tumour growth promotion, since Epo is a pro-survival factor

for cancer cells expressing EpoR (Henke et al., 2003). However, recent studies did not confirm the latter case (Bohlius et al., 2006; Bennett et al., 2010). There seem instead to be a link between excessively high Hb levels and risk of cardiovascular complications (Besarab et al., 1998; Haase, 2013; McCullough et al., 2013): indeed repetitive administration of rhEpo usually lead to high Hb/Hct levels, far above the physiological range necessary to treat anaemia. Additional complications of this treatment regimen are the high cost of therapy, which virtually continues for the entire life of the chronic anaemic patient, and the need for parenteral administration. A large number of companies are currently working on novel erythropoiesis-stimulating agents, including PHD inhibitors, with promising results in rodents and humans (Yan et al., 2010; Flamme et al., 2014). Besides increased compliance of the patients, pre-clinical data showed that PHD inhibitors are capable of reversibly increasing Hb/Hct to more physiological levels compared to rhEpo, without significantly affecting the other HIF target genes tested in the study (Wang et al., 2012; Hong et al., 2013; Flamme et al., 2014).

1.5 Regulation of Epo by humoral factors

Some evidences in literature suggest the possibility for Epo to be regulated through putative humoral factors, independently of tissue hypoxia in the kidney. Despite the fact that some single peptides have been shown to directly regulate Epo *in vitro* or *in vivo*, the precise mechanism of Epo humoral regulation remains clear. It is reasonable to assume that other factors are involved in Epo normoxic/hypoxic transcription, in the negative feedback upon chronic hypoxia and in pathological conditions. REPCs de-differentiation into myofibroblasts could be the reason for anaemia in CKD patients but this hypothesis is not yet proven and regulatory peptides could significantly contribute to Epo impairment in these patients.

The most common hypothesis suggests Epo as a blood volume (BV)-regulating hormone since it is the main factor influencing the number of RBCs, which accounts for approximately 45% of BV. Manoeuvres affecting central venous pressure (mimicking increased BV filling in the right atrium), such as head-down tilt, led to decreased Epo plasma levels (Gunga et al., 1996), while in conditions of blood fluid loss (decreased BV) Epo levels in the plasma have been shown to increase in dogs and humans (Ehmke et al., 1995; Revelli et al., 2013). Moreover, one report showed that intravenous infusion of plasma albumin and physiological solution in healthy humans, thus mimicking an acute increase in BV, led to 14.3% lower Epo plasma levels. Epo concentration remained decreased for 5 h after the injection (Szyguła et al., 1995). All these experiments were

performed at sea level or in water immersion, implying that atmospheric hypoxia cannot be the cause of the changes observed in Epo concentration. Even more interesting, the hypophysis seems to play a role in Epo regulation: hypoxia stimulation of the brain stem (but not of the kidney) in rats led to increased Epo plasma levels and this effect is gone when the animals were nephrectomized or hypophysectomized, suggesting that the brain stem is able to sense O_2 and regulate Epo production in the kidney (von Wussow et al., 2005). Finally, one report described a direct link between ADH/AVP (anti-diuretic hormone/arginine vasopressin) and Epo in rats. Epo plasma levels doubled when rats were treated with ADH in normoxic conditions and blockade of the V1a receptor completely abolished this effect without changes in renal blood flow (Engel and Pagel, 1995).

A second research line focuses on the role of inflammatory mediators on Epo regulation. Inflammation is known to cause anaemia, called anaemia of infection or anaemia of chronic disease, due to several factors: decreased proliferation rate of erythroid progenitors, dysregulation of iron homeostasis and decreased Epo expression. Clinical observation of a blunted erythropoietic response came from patients with autoimmune disease, such as rheumatoid arthritis and inflammatory bowel disease, with acute and chronic infections, including acquired immunodeficiency syndrome (AIDS), and with cancer (Jelkmann, 1998; Weiss and Goodnough, 2005). BFU-E and CFU-E proliferation is impaired in patients with anaemia of infection, possibly because of the growth-inhibitory and pro-apoptotic effect of inflammatory cytokines, among which interferon γ seems to be the most important mediator (Means, 2003). Iron homeostasis dysregulation is a hallmark of inflammatory chronic diseases and is caused by increased uptake and retention of iron by the mononuclear phagocyte system, making iron less available for erythropoiesis. The underlying mechanisms are not fully understood, but up-regulation of iron-related protein such as DMT-1 and ferritin in macrophages has been shown in animal models of inflammation. Again, inflammatory cytokines are the soluble mediators of the effect (Ludwiczek et al., 2003). Epo plasma levels in patients suffering from anaemia of inflammation are not adequate to the degree of anaemia in most of the cases, meaning that Epo concentration is too low for the Hb concentration/Hct observed (Ward et al., 1971; Miller et al., 1990; Camacho et al., 1991). Impaired renal Epo production has been demonstrated in rodents treated with inflammatory stimuli including lipopolysaccharide (LPS) and interleukin-1β (IL-1β), both in normoxia and hypoxia (Jelkmann et al., 1992; Frede et al., 1997). More evidences come from *in vitro* experiments with HepG2 and Hep3B, where LPS, IL-1α, IL-1β and tumour necrosis factor α (TNF-α) showed dose-dependent inhibition of Epo hypoxic induction (Jelkmann et al., 1990). Treatment of macrophages, the cellular source of cytokines, with the anti-inflammatory drug dexamethasone was able to

reverse the effect (Leng et al., 1996). All the above mentioned reports refer to Epo mRNA levels, implying again a regulation at the transcriptional level.

Furthermore, it should be mentioned that factors stabilizing HIFs can have an effect on Epo as well. Several proteins and peptides have been reported to stabilize HIF-α under normoxic conditions but their relevance for Epo regulation is unknown (Wenger et al., 2005). Among the "Epo normoxic regulators", sexual hormones are the most well studied and have been proposed to be the cause of gender-related differences in Hct. Indeed, healthy adult women display 12% lower Hb concentration in venous blood than age and race matched men (Murphy, 2014), but erythropoietin plasma levels do not seem to vary between sexes (Jelkmann and Wiedemann, 1989). Sexual hormones administration to human subjects does affect Hb concentration in the blood; in particular estrogen treatment lowers the Hct, while androgen treatment increases it. The steroid hormone effect on erythropoietic progenitors in the bone marrow is known and partially accounts for sex-related differences in RBC mass (Murphy, 2014). Nevertheless, a series of evidences speak for an effect of steroid hormones on renal *EPO* transcription as well. Estrogen treatment of Hep3B culture decreased Epo-reporter assay activity and protein levels (Mukundan et al., 2004) and estrogen administration to hypoxic rats attenuated Epo synthesis (Mukundan et al., 2002). On the other hand, androgen therapy in human subjects increased Epo plasma and urine levels (Alexanian, 1969), suggesting again a direct effect of sex hormones on transcription of the *EPO* gene. Moreover, aneprhic or ESRD patients do not show gender-related differences in Hct values (Neff et al., 1981) and do not respond with increased Epo production to androgen treatment (Teruel et al., 1996). Collectively, these observations point in the direction of a direct effect of sex hormones on Epo production in the kidney, at the transcriptional level if considering the *in vitro* experiments.

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2. Aims of the thesis

Erythropoietin (Epo) is the main humoral factor regulating red blood cells homeostasis and is transcriptionally regulated in a very thigh manner by a variety of mechanisms. Although it is often used as a paradigm for hypoxia-induced genes, several aspects of Epo regulation remain unclear. In this PhD work, we were interested in using the currently available Epo-expressing cell models in order to study *EPO* transcriptional regulation at different levels.

I. The chromosomal position and function of the element responsible for renal Epo inducibility (the kidney-inducible element, KIE), as well as the maintenance of the extremely high tissue specificity of Epo expression represent the main focus of the thesis. To this aim, we generated a series of reporter constructs containing different *EPO* flanking regions and tested their activity in Epo-expressing and non Epo-expressing cell lines. This approach was combined with transient or stable knockdown/overexpression of various candidates playing a role in the hypoxia-signalling cascade in order to dissect their role in Epo hypoxic regulation. Finally, the cluster regularly interspace short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) technique was introduced as innovative approach to study the remote Epo enhancer elements in cell culture.

II. Novel mutations in Egl9 homolog 1 (*EGLN1*) gene associated with secondary congenital erythrocytosis were identified by the group of Dr. Betty Gardie (Nantes, France). As part of a collaborative effort the functional effect of these mutations on the activity of the encoded protein (prolyl-4-hydroxylase domain 2, PHD2) was characterised.

III. In order to identify putative humoral factors regulating the von Hippel-Lindau protein/prolyl-4-hydroxylase domain/hypoxia-inducible factor (pVHL/PHD/HIF) pathway, and potentially *EPO* transcription as well, a number of selected peptides were tested for their effect on Epo mRNA levels. Moreover, a library of bioactive peptides isolated from haemofiltrates of patients with chronic renal failure generated in the lab of Prof. W.G. Forssmann (Hannover, Germany) was screened for the effect on HIF-dependent reporter cell lines.

3. Manuscript I: A novel distal upstream hypoxia response element regulating oxygen-dependent erythropoietin gene expression

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Running head: A novel distal *EPO* regulating element

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Erythropoietin (Epo) is the main humoral regulator of erythropoiesis. During development Epo production switches in a species-specific manner from the liver to the kidneys which account for ~90% of total Epo synthesis in the adult. Renal Epo is produced by peritubular interstitial cells with fibroblastic and neuronal features, located in the juxtamedullary cortex (Maxwell et al., 1993; Obara et al., 2008; Wenger and Hoogewijs, 2010; Souma et al., 2013). These cells respond to a decrease in tissue oxygen partial pressure by hypoxia-inducible transcription factor-2 (HIF-2)-dependent induction of Epo synthesis (Kapitsinou et al., 2010). DNA sequences required for oxygen-dependent regulation of the *EPO* gene are different in liver and kidney. Under physiologic conditions, *EPO* gene expression is controlled by elements located within 0.4 kb of the 5' and 0.7 kb of the 3'-flanking region in the liver, while an essential regulatory element for renal Epo expression resides between -14 and -6 kb in the distal 5'-region (Semenza et al., 1990; Köchling et al., 1998). The liver-inducible element consists of a proximal downstream enhancer which synergizes with the minimal promoter region to achieve up to 100-fold transcriptional induction (Blanchard et al., 1992). Detailed analysis of this conserved 50 bp 3'-enhancer revealed a tripartite *cis*-regulatory structure with a consensus HIF binding site (HBS, ACGTG) and a CACA repeat downstream of the HBS (Semenza and Wang, 1992). The latter element is necessary, but not sufficient for hypoxia inducibility of the 3'-enhancer. However, so far no protein binding to this element has been defined. While the Epo 3'-enhancer is well characterized and has been confirmed to be both essential and sufficient for liver-specific Epo expression in mice beyond embryonic day 14.5 (Suzuki et al., 2011), the kidney-inducible element (KIE) remains unexplored. In the present study we functionally analyzed a distal upstream hypoxia response element (HRE) which confers oxygen regulated Epo transcription and presumably represents the hitherto uncharacterized KIE.

We used the ENCODE data integrated in the UCSC Genome Browser (http://genome.ucsc.edu/) to identify a distal conserved putative HBS in the Epo 5'-region, located within a DNase I hypersensitivity site (DSS) observed in 9 different non-Epo producing cell lines and strongly conserved in multiple vertebrate species (figure 1A). This distant locus, 9248 bp upstream of the Epo transcription start site, contains a putative HBS and is flanked by the most frequently neighboring residues observed around the consensus HBS (Schödel et al., 2011) and a CACA repeat 8 bp upstream, very similar to the established 3'-HRE. The striking overlap of this region with a previously reported anemia-inducible DSS in liver and kidney of anemic Epo transgenic mice (Köchling et al., 1998) prompted us to perform a detailed functional analysis. Two Epo-expressing (Hep3B and Kelly, figure 1B) and two non-expressing (HeLa and HK-2) human cell lines were transfected with firefly luciferase reporter gene constructs under the control of the

exogenous SV40 promoter and a 100 bp fragment encompassing the novel candidate 5'-HRE, containing either wild-type or mutant HBS and CACA elements. The isolated distal 5'-HRE conferred robust hypoxic reporter gene induction in all cell lines tested (figure 1C), which was abrogated upon mutation of either the HBS or the CACA sequence (figure 1D).

We next combined the endogenous Epo minimal promoter (117 bp) with the distal 5'-HRE of either 100 bp, 300 bp (covering the entire conserved DSS region), or 3 kb length as indicated in Figure 2A. All three constructs, but not the Epo minimal promoter alone, increased reporter gene activity in hypoxic Hep3B cells (figure 2B). No further induction was observed by inclusion of the 3'-HRE (126 bp), suggesting that at least under our experimental conditions the 5'-HRE and the 3'-HRE do not cooperate. Because the 3 kb containing fragment did not further increase the hypoxic response it was excluded from later analyses.

To analyze HIFα isoform transcriptional selectivity, reporter gene constructs were co-transfected with constitutively active (double proline mutant) HIF-1α or HIF-2α overexpressing plasmids. Reporter assays using the plasminogen activator inhibitor 1 (PAI-1) and carbonic anhydrase IX (CAIX) promoters as well as pH3SVL served as controls for HIFα overexpression. HIF-2α enhanced transactivation of the Epo promoter substantially better than HIF-1α (figure 2C), in line with the previously reported dominant role of HIF-2α in hypoxia-inducible Epo transcription in vitro (Warnecke et al., 2004). Interestingly, no HIFα isoform specificity was observed in reporter genes driven by the heterologous SV40 promoter (figure 2D), indicating a crucial role of the endogenous Epo minimal promoter in HIFα isoform selectivity. Reporter gene assays were repeated in small interfering (si)RNA-mediated HIFα-deficient Hep3B cells, as described before (Wollenick et al., 2012). Both HIF-1α and HIF-2α siRNA reduced 5'-HRE dependent luciferase activity in hypoxic Hep3B cells (figure 2E). Rather unexpectedly, both Epo 5' and 3'-HRE dependent luciferase activity was more affected by depletion of HIF-1α than HIF-2α. This might be partially explained by less efficient knockdown of HIF-2α, as reflected by the PAI-1 and pH3SVL reporter activity and HIF-2α mRNA expression levels (figure 2E and F). Alternatively, knockdown of HIF-1α might decrease hypoxic prolyl hydroxylase domain protein 3 (PHD3) induction, which has been shown to preferentially target HIF-2α (Appelhoff et al., 2004). Notably, hypoxia-inducible Epo mRNA expression was not entirely HIF-2 dependent in hypoxic Hep3B cells (figure 2F).

To analyze HIFα DNA binding to the endogenous sites, chromatin immunoprecipitation (ChIP) assays were performed using either anti-HIF-1α (5 µg, Ab2185; Abcam) or anti-HIF-2α (5 µg, Ab199; Abcam) antibodies in hypoxically treated Hep3B cells, as described before (Wollenick et

al,. 2012). The PAI-1 and Epo 3'-HRE were employed as positive controls, and regions 1 kb upstream and downstream of the 5'-HRE served as negative controls. ChIP analysis revealed hypoxia-inducible HIF-2α, but not HIF-1α enrichment at the 5'-HRE or at any other of the tested 5' loci (figure 2G, left panel). In contrast, both HIF-1α and HIF-2α bound to the Epo 3'-HRE and the known PAI-1 HRE (figure 2G, right panel).

Collectively, our results demonstrate the identification of a functional HIF-2α dependent distal 5'-HRE regulating Epo transcription. The lack of a renal cell culture system capable of hypoxia-inducible Epo expression precluded further validation of the kidney-specificity of this 5'-HRE. Additional distal elements contributing to kidney-specific Epo expression are likely to exist, because a BAC covering more than 180 kb flanking Epo regulatory regions is required to fully recapitulate endogenous Epo expression in transgenic mice (Obara et al., 2008; Yamazaki et al., 2013). Our study is the first to functionally analyze the enigmatic KIE and provides the basis for further investigations on the cooperation between other putative distal and proximal regions in regulating kidney-specific inducible Epo expression.

Authorship and Disclosures

FS, SS, LC, TO and DH designed research, performed research and analyzed data; IAR and MK performed research and analyzed data; CJH provided tools; CD and JF provided tools and critically reviewed the paper; RHW designed research and critically reviewed the paper; DH supervised the study and wrote the paper. The authors report no potential conflicts of interest.

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Figure 1

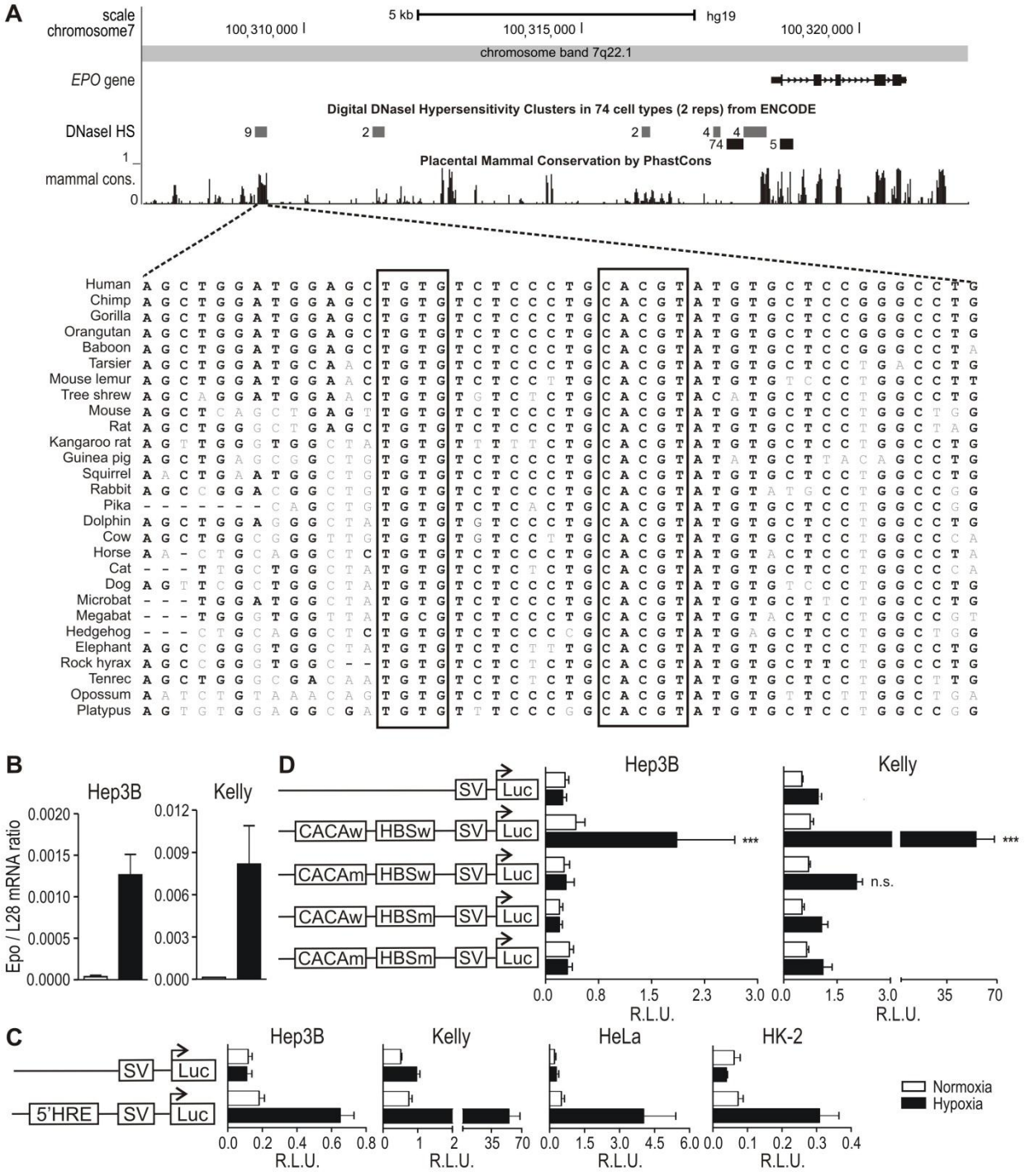

Figure 1 Location of a functional distal HRE in the Epo 5' regulatory region. **A.** UCSC Genome Browser output (hg19) of the Epo genomic and 5' upstream region. Shown are the ENCODE DSS clusters and mammalian PhastCons conservation tracks with a closer view of the region in 28 vertebrates extracted using the 46-MULTIZ whole-genome multiple alignment algorithm. **B.**

Epo mRNA levels in human Hep3B and Kelly cells were measured by RT-qPCR and normalized to ribosomal protein L28 mRNA levels. **C.** Both cell types ($3x10^5$ cells) were co-transfected with the indicated SV40-driven firefly luciferase reporter gene plasmids (500 ng) and a *Renilla* luciferase control plasmid (5 ng, Promega) in a six-well format by polyethylenimine (PEI). 24 h post-transfection, cells were incubated for another 24 h under normoxic or hypoxic conditions (0.2% O_2). Luciferase activities of triplicate wells were determined using the Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). All results are displayed as ratios of firefly to *Renilla* relative light units (R.L.U.). **D.** Cells were co-transfected with pGL3p Epo 5'-HRE wild-type (w), pGL3p Epo HBS mutated (m) (ACGTG to AAAAG), pGL3p Epo CACA mutated (CACA to AAAA) or pGL3p Epo HBSm, CACAm and a *Renilla* luciferase control plasmid, and reporter gene experiments performed as in (**D**). **B-D**. All data are expressed as mean ± SEM of 3 independent experiments and statistical analyses were performed with one-way ANOVA and Tukey correction for multiple comparisons (***$p < 0.001$, n.s. = not significant).

Figure 2

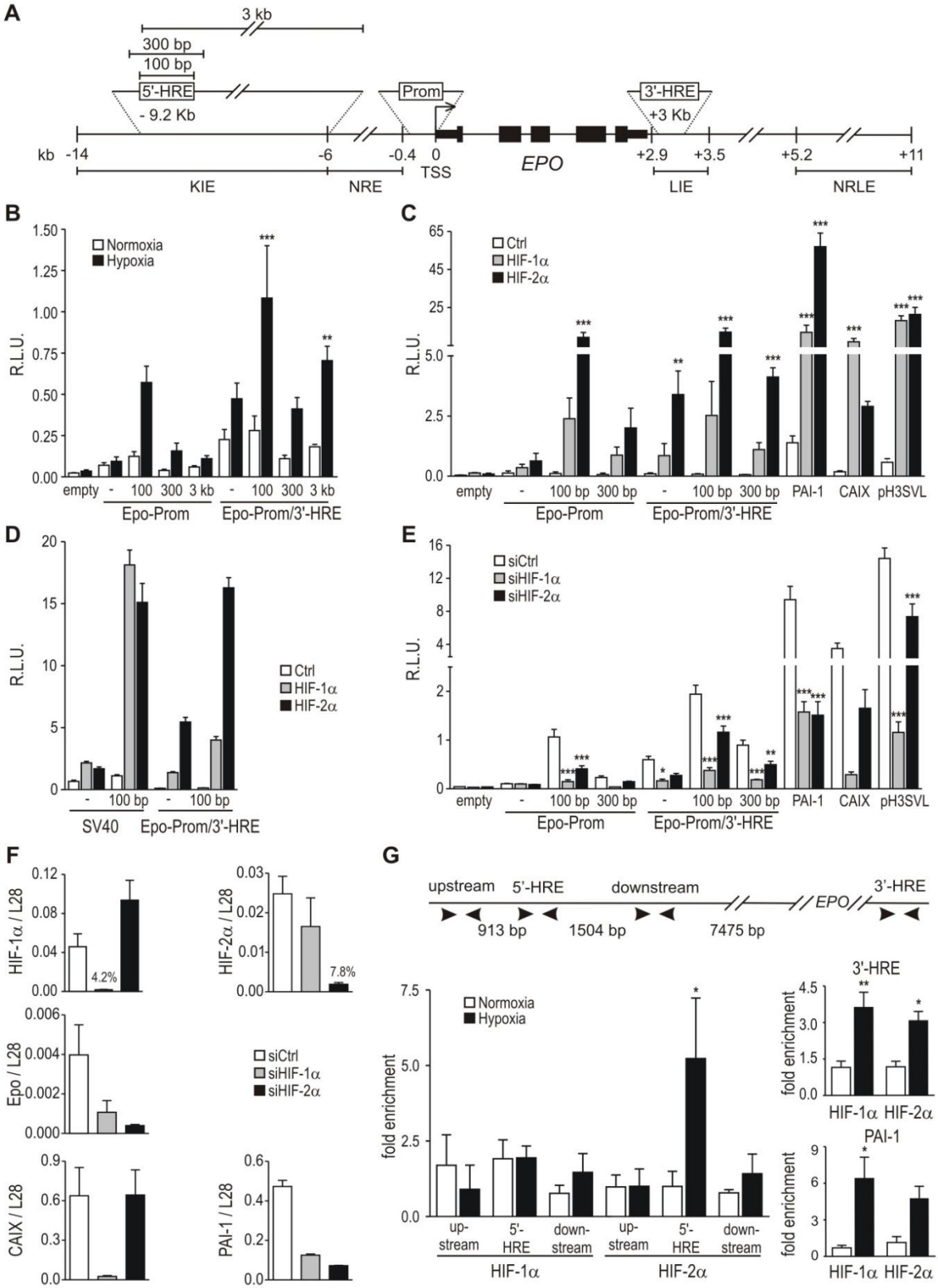
A
3 kb
300 bp
100 bp
5'-HRE
- 9.2 Kb
Prom
3'-HRE
+3 Kb
kb -14
-6
-0.4 0
TSS
EPO
+2.9
+3.5
+5.2
+11
KIE
NRE
LIE
NRLE
B
Normoxia
Hypoxia
R.L.U.
empty
100
300
3 kb
Epo-Prom
Epo-Prom/3'-HRE
C
Ctrl
HIF-1α
HIF-2α
100 bp
300 bp
PAI-1
CAIX
pH3SVL
D
SV40
E
siCtrl
siHIF-1α
siHIF-2α
F
HIF-1α / L28
4.2%
HIF-2α / L28
7.8%
Epo / L28
CAIX / L28
PAI-1 / L28
G
upstream
5'-HRE
downstream
3'-HRE
EPO
913 bp
1504 bp
7475 bp
fold enrichment
up-stream
down-stream

Figure 2 HIF-2α dependent hypoxic regulation of the Epo distal enhancer element. **A.** Scheme depicting the constructs used. The numbers are relative to the transcriptional start site (TSS). KIE, kidney-inducible element; NRE, negative regulatory element; LIE, liver-inducible element; NRLE, negative regulatory liver element. **B.** Dual luciferase analyses using the Epo minimal promoter (117 bp) and several indicated extensions of the distal 5'-HRE alone or in combination with the 3'-HRE. All reporter gene experiments were performed as in Figure 1. **C.** The indicated vectors were co-transfected with HIF-α or empty expression vectors into Hep3B cells. CAIX and PAI-1 promoter constructs were used as controls for HIF-1α and HIF-2α specificity, respectively. pH3SVL is controlled by a concatamerized non-HIFα isoform specific HRE and an SV40 promoter. **D.** Comparison between SV40-driven and Epo promoter-driven luciferase expression, following HIFα overexpression in Hep3B cells. **E.** Effect of siRNA-mediated HIFα knockdown on the indicated vectors in Hep3B cells. **F.** HIF-1α, HIF-2α, Epo, PAI-1 and CAIX mRNA levels were measured by RT-qPCR and normalized to ribosomal protein L28 mRNA levels. **G.** Scheme depicting the different primer regions used for ChIP experiments. ChIP of normoxic or hypoxic (0.2% O_2, 24 h) Hep3B cells using antibodies directed against HIF-1α or HIF-2α. The amount of co-precipitated chromatin derived from the human Epo 5' regulatory region, its upstream and downstream regions, and the Epo 3'-HRE was determined by qPCR. The PAI-1 promoter was used as additional positive control. Mean values ± SEM of 3 (**B-F**) or 4 (**G**) independent experiments are shown and statistical analyses were performed with one-way ANOVA and Tukey correction for multiple comparisons (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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4. Manuscript II: Distinct deregulation of the hypoxia inducible factor by PHD2 mutants identified in germline DNA of patients with polycythemia

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Abstract

Background Congenital secondary erythrocytoses are due to deregulation of hypoxia inducible factor resulting in overproduction of erythropoietin. The most common germline mutation identified in the hypoxia signaling pathway is the Arginine 200-Tryptophan mutant of the von Hippel-Lindau tumor suppressor gene, resulting in Chuvash polycythemia. This mutant displays a weak deficiency in hypoxia inducible factor α regulation and does not promote tumorigenesis. Other von Hippel-Lindau mutants with more deleterious effects are responsible for von Hippel-Lindau disease, which is characterized by the development of multiple tumors. Recently, a few mutations in gene for the prolyl hydroxylase domain 2 protein (*PHD2*) have been reported in cases of congenital erythrocytosis not associated with tumor formation with the exception of one patient with a recurrent extra-adrenal paraganglioma.

Design and Methods Five *PHD2* variants, four of which were novel, were identified in patients with erythrocytosis. These *PHD2* variants were functionally analyzed and compared with the *PHD2* mutant previously identified in a patient with polycythemia and paraganglioma. The capacity of *PHD2* to regulate the activity, stability and hydroxylation of hypoxia inducible factor α was assessed using hypoxia-inducible reporter gene, one-hybrid and in vitro hydroxylation assays, respectively.

Results This functional comparative study showed that two categories of *PHD2* mutants could be distinguished: one category with a weak deficiency in hypoxia inducible factor α regulation and a second one with a deleterious effect; the mutant implicated in tumor occurrence belongs to the second category.

Conclusions As observed with germline von Hippel-Lindau mutations, there are functional differences between the *PHD2* mutants with regards to hypoxia inducible factor regulation. *PHD2* mutation carriers do, therefore, need careful medical follow-up, since some mutations must be considered as potential candidates for tumor predisposition.

Introduction

Secondary erythrocytosis is due to external factors such as increased production of erythropoietin, the origin of which is variable and may result from germline mutations in genes encoding factors involved in the oxygen-sensing pathway. The primary cellular component implicated in oxygen homeostasis is the hypoxia-inducible transcription factor (HIF). HIF operates as a heterodimer composed of a constitutively expressed beta subunit, also known as aryl hydrocarbon receptor nuclear translocator, and an alpha subunit (1α, 2α or 3α) that is tightly regulated by oxygen via post-translational modification. The prolyl-4-hydroxylase domain (PHD) enzymes hydroxylate proline residues located in the oxygen-dependent degradation (ODD) domain of HIF-α. This hydroxylation allows the binding of the von Hippel-Lindau protein (pVHL), the substrate recognition subunit of an E3 ubiquitin ligase complex that induces ubiquitination and subsequent degradation of HIF-α by the proteasome (Pouyssegur et al., 2006; Kaelin Jr, 2008). In the absence of oxygen, HIF-α is stabilized, heterodimerizes with HIF-1β and induces expression of hundreds of genes involved in cell survival, angiogenesis, erythropoiesis and cell proliferation (Wenger et al., 2005; Pouyssegur et al., 2006). There is some restricted target gene specificity depending on the HIF-α subunit of the HIF-α/β heterodimeric transcription factor. For example, renal and hepatic erythropoietin is regulated by the HIF-2α subunit *in vivo* (Rosenberger et al., 2002; Warnecke et al., 2004; Rankin et al., 2007; Wenger and Hoogewijs, 2010). Germline mutations in genes involved in the HIF pathway have been reported in association with syndromes that predispose patients to both neoplasms and/or congenital secondary erythrocytosis (Richard et al., 2004). The most frequent mutations involve the *VHL* tumor suppressor gene. Heterozygous germline mutations in this gene are responsible for von Hippel-Lindau (VHL) disease, an autosomal dominant condition predisposing to multiple tumors including central nervous system and retinal hemangioblastomas, clear-cell renal cell carcinoma, pheochromocytomas and pancreatic endocrine tumors (Richard et al., 2004). Established correlations between genotype and phenotype predict the risk of paraganglioma/pheochromocytoma, with *VHL* deletions or truncating mutations being associated with a low risk (VHL type 1) and *VHL* missense mutations being associated with a high risk (VHL type 2) (Maher et al., 1996; Nordstrom-O'Brien et al., 2010).

In addition, a homozygous 598C>T (R200W) *VHL* germline mutation has been shown to account for Chuvash congenital polycythemia, an autosomal recessive disease, endemic in the Chuvash Autonomous Republic of the Russian Federation (Ang et al., 2002). Homozygous carriers of the R200W-*VHL* germline mutation do not develop tumors but instead have Chuvash congenital polycythemia due to high levels of erythropoietin (Ang et al., 2002). The lack of tumor

development in this disorder is due to a weak defect of the mutation in terms of its HIF-α regulation (leading to delayed ubiquitination) because of its localization outside pVHL functional domains (Ang et al., 2002). Other homozygous and compound heterozygous polycythemia-associated *VHL* mutations have also been reported (Nordstrom-O'Brien et al., 2010). Functional studies of some of these mutants have shown a weak to undetectable defect of HIF-1α regulation (unpublished data).

Recently, a similar phenotype of high erythropoietin-associated polycythemia without associated tumors has been reported in carriers of heterozygous germline mutations in the *PHD2* and *HIF-2A* genes (Percy et al., 2006; Percy et al., 2007; Al-Sheikh et al., 2008; Gale et al., 2008; Martini et al., 2008; Percy et al., 2008; Percy et al., 2008; van Wijk et al., 2010), with the exception of one patient carrying a H374R-PHD2 mutation (Ladroue et al., 2008). This particular patient simultaneously developed congenital secondary erythrocytosis and recurrent paraganglioma, a tumor originating from neural crest cells similar to pheochromocytoma but with an extra-adrenal localization (Ladroue et al., 2008). The analysis of the tumor showed a loss of heterozygosity including the wild-type *PHD2* allele, suggesting a potential tumor suppressor role of *PHD2*.

PHD2 and VHL act in concert to regulate HIF-α. Based on the observation that VHL mutation carriers display different phenotypes depending on the relative capacity of the *VHL* mutants to regulate HIF, we sought to define the genotype-phenotype relationship regarding the capacity of *PHD2* mutants to differentially regulate HIF-α. Here we report a functional study comparing five *PHD2* variants associated with isolated congenital secondary erythrocytosis with the mutation identified in a patient with recurrent paraganglioma.

Design and Methods

Patients and mutation screening

Thirty-four patients who did not fulfill World Health Organization diagnostic criteria for polycythemia vera were investigated. The local ethics committee of Kremlin Bicetre Hospital approved the study and all patients provided written informed consent. Blood samples were collected from all patients and germline DNA was extracted and analyzed by direct sequencing (Ladroue et al., 2008). DNA from the blood of healthy donors of Caucasian origin was used as a control.

Assay of hypoxia inducible factor transcriptional activity

In order to assay HIF transcriptional activity, dual luciferase assays were performed in Hek293T cells as described previously (Ladroue et al., 2008). A pGL3promoter vector expressing luciferase under the control of hypoxia response elements (Dayan et al., 2006) was used. Cells were exposed to hypoxic conditions (1% O_2) for 4 h before extraction.

Assay of hypoxia inducible factor stability

HeLa cells were transiently co-transfected with increasing amounts (50-200 ng) of an expression vector encoding the HIF-2α ODD domain (amino acids 404-569), fused to yeast Gal4 DNA-binding domain and Herpes simplex VP16-derived transactivation domain, together with the Gal4-response element driven firefly luciferase reporter, pGRE5xE1b (125 ng), and a *Renilla* luciferase control plasmid (4 ng) (Köditz et al., 2007). Twenty-four h post-transfection, cells were cultured under either normoxic (20% O_2) or hypoxic (1% O_2) conditions for an additional 16 h, and firefly luciferase activity was determined and normalized to *Renilla* luciferase activity.

Hydroxylation assay

PHD2 and HIF-ODD proteins (plasmid pcDNA3-HA-Gal4-HIF-1α-ODD was a generous gift from WG Kaelin Jr, Dana-Farber Cancer Institute, Boston, USA) (Ohh et al., 2000) were produced in wheat germ extract *in vitro* using the TnT transcription-translation kit (Promega). The hydroxylation reaction was carried out by mixing PHD2 and HIF-1α ODD (amino acids 536-652) proteins in a reaction buffer containing co-factors (Fe2+, ascorbic acid and 2-oxoglutarate) as described previously (Huang, Zhao et al. 2002). The samples were incubated at 30°C, collected at different time points, and immunoblotted with anti-HA (Tebu) and anti-HIF-1α (Pro 564)-OH (Cell-Signaling Technology) antibodies.

Results

We sequenced the *PHD2* gene on germline DNA from a series of Caucasian patients with unexplained polycythemia associated with normal or elevated serum erythropoietin levels (figure 1A). Four novel heterozygous sequence variants were identified in the *PHD2* gene [c.G471C, p.Gln157His (Q157H); c.C599A, p.Pro200Gln (P200Q); c.G760C, p.Asp254His (D254H); c.C1192T, p.Arg398X (R398X)] as well as the already described c.G1112A, p.Arg371His (R371H) mutation (Percy et al., 2007) (Figures 1A and B). Genetic testing was performed on

available parents and relatives of the *PHD2* mutation carriers, but no mutation was found except in the mother of the *PHD2*-R398X carrier who presented characteristics of polycythemia (figure 1A). She harbors the *PHD2* mutation but with a mosaic status as demonstrated by the reduced height of the peak on the sequence chromatogram (figure 1B). The low proportion of the mutated allele was confirmed by a quantitative allele-specific oligonucleotide method (data not shown).

Concerning the other families, only a few relatives were available for further genetic and clinical investigations but there was no history of familial polycythemia. Briefly, the brother, the sister and the son of patient #2295 and the father of patient #2403 agreed to genetic testing and were not carriers of *PHD2* mutation. Other parents had died previously, of known causes in two cases: esophageal cancer for the mother of patient #2403 and colon cancer for the mother of patient #2412. Finally, patient #0424 was an adopted child, without children.

The frequencies of the different variants were evaluated in a control population (figure 1C). Only the Q157H variant was found and was, therefore, classified as a polymorphism. Analysis of amino acid conservation supports this conclusion, as the Q157 amino acid is not conserved either between species or within the PHD protein family (PHD1 and 3) (online supplementary table S1). In contrast, amino acids P200 and R371 are highly conserved and the D254 amino acid is fully conserved, similar to H374 described in our previous study (Ladroue et al., 2008). As far as concerns the location of the amino acids in the protein, D254, like H374, is part of the PHD2 catalytic site (McDonough et al., 2006). The P200 amino acid is located within the nuclear localization signal (NLS) implicated in the shuttling of PHD2 between the cytoplasm and the nucleus which plays a crucial role in HIF regulation (Steinhoff et al., 2009). We tested the shuttling of the P200Q mutation by immunofluorescence but we did not observe any impact of the mutation on the capacity of PHD2 to shuttle (data not shown).

We next studied the *PHD2* mutants using a one-hybrid reporter assay based on the capacity of PHD2 to induce HIF-α protein instability. Cells were co-transfected with expression vectors containing the different PHD2 mutants (50 to 200 ng), the oxygen-dependent degradation domain of HIF-2α (HIF-2α-ODD) fused to yeast Gal4 DNA-binding domain and Herpes simplex VP16-derived transactivation domain, together with a reporter vector expressing luciferase under the control of a Gal4-response element. In this assay, luciferase expression reflects the stability of HIF-2α ODD. A trend similar to that in the previous test was obtained: there was no detectable effect of the P200Q and R371H substitutions on PHD2 activity (which was comparable to that of the wild-type PHD2) and there was total abolition of the D254H mutant activity (comparable to

that of the truncated PHD2 variant R398X, figure 2B). Identical results were obtained using a HIF-1α ODD stability reporter (data not shown). The experiment was repeated with the P200Q-PHD2 mutant in oxygen-regulated erythropoietin- expressing cells (including the human hepatoma cell line Hep3B and renal erythropoietin-producing cells, a new cell model isolated from the tumor-free tissue of a patient with renal carcinoma) but no significant difference was observed (data not shown).

We next performed a sensitive *in vitro* assay in order to test the ability of the PHD2 mutants to hydroxylate HIF-1α in a time-dependent manner. In this assay, *in vitro*-translated PHD2 proteins were mixed with HIF-1α ODD in the presence of co-factors necessary for the hydroxylation reaction. The capacity of the different PHD2 mutants to hydroxylate HIF-1α was measured by immunoblotting using an antibody specific for the hydroxylated HIF-1α ODD (HIF-OH, Figure 3A). The H374R and D254H substitutions totally impaired HIF-1α hydroxylation (figure 3A). By contrast the R371H mutant behaved like the wild-type PHD2 and the P200Q mutant, although capable of hydroxylating HIF-1α (figure 3A), showed a reproducible and consistent delay (figure 3B).

Discussion

Taken together, these results show that *PHD2* mutations can be divided into several different classes in terms of their effects on HIF regulation. Genotype/phenotype correlations cannot be established for *PHD2* mutations because they are rare events that have been reported in only ten families to date (including those in the present study), in contrast to the 945 families described with a *VHL* mutation (Nordstrom-O'Brien et al., 2010). In addition, the parents of the *PHD2* mutation carriers reported in the literature were either dead or not available for further genetic and clinical investigations (including one parent who died of esophageal cancer (Percy et al., 2006)). In our study, only one parent was genetically tested and diagnosed as a mosaic carrier which prevents any conclusion regarding the developed phenotype. Nonetheless, regarding the close functional relation between VHL and PHD2 in the regulation of HIF and the implication of the HIF pathway in the genesis of pheochromocytoma (Eisenhofer et al., 2004; Dahia et al., 2005; Pollard et al., 2006; Favier et al., 2009), we can hypothesize similarities between the various types of mutants and raise the question of a possible risk of development of paraganglioma/pheochromocytoma in *PHD2* mutation carriers. Subjects with one category of mutation (P200Q and R371H) display features similar to those with the VHL-R200W mutation

responsible for Chuvash polycythemia without any increased risk of neoplasia (Ang et al., 2002). Like the VHL-R200W mutant, which is located outside functional domains, the PHD2-P200Q and R371H mutations are not located in the catalytic domain of the enzyme and have a moderate impact on HIFα regulation. In addition, the VHL-R200W mutant only induces delayed ubiquitination of HIFα which may be comparable to the delayed hydroxylation of HIFα observed with the PHD2-P200Q mutant. Interestingly, Pro200 is only one residue N-terminal to Cys201 which has been shown to chelate zinc and cadmium ions, providing evidence for the existence of a second metal binding site on PHD2 (Mecinović et al., 2008; Mecinović et al., 2009). This Cys201 affects PHD2 hydroxylation activity and appears to be implicated in redox signaling *in vitro (Nytko et al., 2011).* The very close location to the functionally important Cys201 residue could be the cause of the delayed hydroxylation of HIF-1α by the PHD2-P200Q mutant. Intriguingly, concerning the confirmed R371H mutant, the previously reported loss-of-function effect of this mutant (Percy et al., 2007) could not be confirmed by any of the three tests of our study. The R371H mutation segregates with erythrocytosis in two different families (described herein and by Percy *et al. (Percy et al., 2007)*) and is unequivocally involved in this pathology. We currently cannot explain why, in our hands, the R371H mutation failed to abolish PHD2 catalytic activity. The PHD2 expression vectors were re-sequenced and their functions confirmed by immunoblotting. Parallel experiments with other PHD2 mutants confirmed the validity of our assays. For this category of mutants which have a moderate impact on HIFα regulation, we cannot rule out potential indirect regulation on the oxygen sensing pathway via PHD2-interacting proteins. Indeed, during the past decade a large number of PHD2-interacting proteins have been discovered, including both upstream regulators and downstream targets of PHD2, substantially increasing the complexity of the PHD/HIF oxygen-sensing regulation pathway (Wenger and Hoogewijs, 2010). Moreover, PHD2 has been reported to have hydroxylation-independent gene regulatory functions (Chan et al., 2009; Shao et al., 2009; Bordoli et al., 2011).

Another category, including the PHD2-R398X mutation and three other *PHD2* truncated mutations described previously (Al-Sheikh et al., 2008), can be compared to the *VHL* truncation mutations (VHL disease type 1) which are not associated with the development of pheochromocytomas. Subject with these first two categories could be considered at low risk of developing paraganglioma/pheochromocytoma.

A last category could be compared to the *VHL* missense mutations involved in VHL disease type 2, associated with a high risk of paraganglioma/pheochromocytoma. This category includes the previously described PHD2-H374R mutation identified in a 43-year-old patient with paraganglioma. A loss of the *PHD2* wild-type allele was demonstrated in the patient with this

tumor, arguing for a tumor suppressor role of *PHD2 (Ladroue et al., 2008)*. No *PHD2* mutations have been identified in other series of patients affected by pheochromocytomas (73 patients with hereditary paraganglioma and pheochomocytoma syndrome, Gimenez-Roqueplo, Hôpital Européen Georges Pompidou, Paris, *unpublished data*) and/or renal carcinoma (Astuti et al., 2011), but the risk that germline *PHD2* mutation carriers have of developing tumors should not be underestimated. Indeed, like His374, Asp254 is highly conserved, and located in the catalytic site of PHD2 (McDonough et al., 2006). Moreover, the D254H mutation results in a severe loss of function. Therefore, although no cases of paraganglioma or pheochromocytoma have yet been detected in the D254H-*PHD2* mutation carrier, this mutation may be considered as a potential candidate for tumor predisposition.

In conclusion, using three different approaches we demonstrated that distinct *PHD2* mutations have differential effects on HIF regulation. We suggest that, by analogy to *VHL* mutations, carriers of particular *PHD2* mutations may be prone to tumor development. These patients would then require screening for tumor prevention and early detection.

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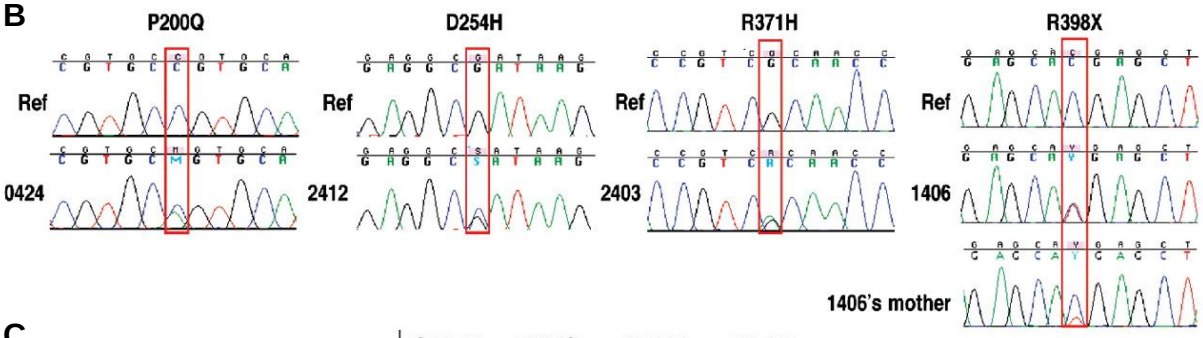
Figure 1

A

Patient number	Mutation (nucleotide)	Mutation(protein)	Age	Age at the diagnosis of polycythemia	Sex	Ht (%) (Nm=40-52) (Nf=37-47)	Hb (g/dL) (Nm=13-18) (Nf=12-15)	RBC (10^6/mm^3) (Nm=4.2-5.7) (Nf=4.2-5.2)	EPO (mU/mL) (N=5-25)
8416	c.G471C	p.Gln157His (**Q157H**)	44	43	m	57.2	20.2	NA	24
0424	c.C599A	p.Pro200Gln (**P200Q**)	34	22	m	56	17.9	5.9	90
2412	c.G760C	p.Asp254His (**D254H**)	48	25	m	57.2	19.2	6.3	1.5-2 x N
2403	c.G1112A	p.Arg371His (**R371H**)	25	17	m	56.7	19.1	6.4	N
1406	c.C1192T	p.Arg398X (**R398X**)	41	26	m	53.8	19.3	5.9	6.5
1406's mother	c.C1192T	p.Arg398X (**R398X**)	67	64	f	49.5	16.1	5.2	NA
2295*	c.A1121G	p.His374Arg (**H374R**)	52	30	m	61.6	20.2	6.2	18

B



C

	Q157H	P200Q	D254H	R371H
Number of sequenced controls	173	173	188	133
Number of identified substitutions	5	0	0	0
Frequency	3%	0%	0%	0%

Figure 1 Identification of *PHD2* mutations in patients with polycythemia. **A.** Table of patients diagnosed with erythrocytosis and a *PHD2* variation. Ht: hematocrit; Hb: hemoglobin; RC: number of red cells; EPO: erythropoietin; m: male; f: female; N: normal; NA: not available; *: previously described (Ladroue, Carcenac et al. 2008). **B.** Sequence chromatogram of *PHD2* in the area of the mutated nucleotide. Wild-type DNA was used as reference (Ref) (top) and compared to germline DNA of the patient (bottom). Only one relative of a patient was available for genetic testing (the mother of the *PHD2*-R398X carrier). She harbors the *PHD2* mutation but with a mosaic pattern as demonstrated by the reduced height of the peak on the sequence chromatogram. **C.** Frequency of the *PHD2* variations in a control population.

Figure 2

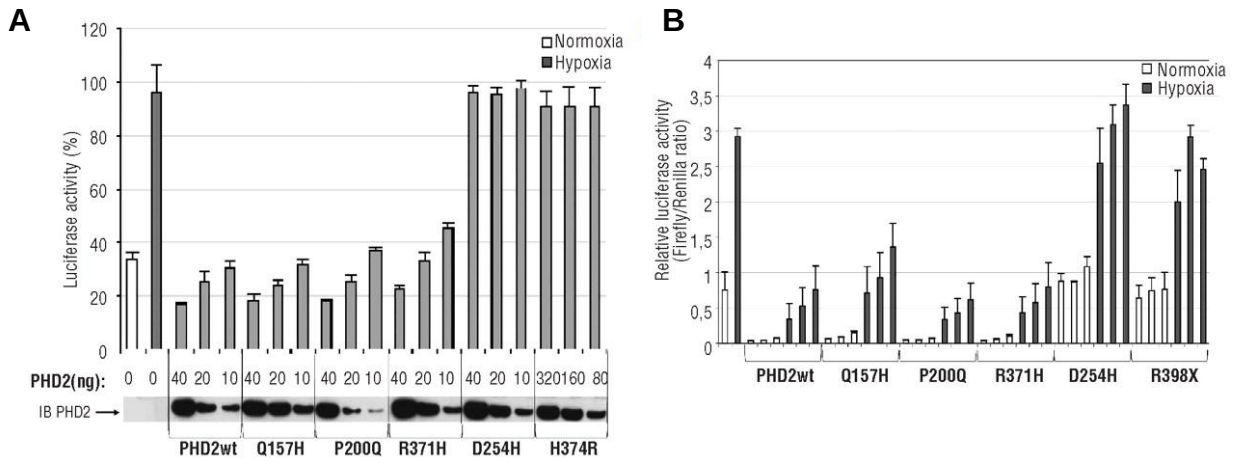


Figure 2 Functional study of *PHD2* mutants using luciferase reporter assays. **A.** PHD2-dependent regulation of endogenous HIF in an assay based on a hypoxia response element reporter gene. Cells were co-transfected in a 12-well format with various amounts of pcDNA3-HA-PHD2 expression vectors (to enable the expression of the same amount of PHD2 proteins) in addition to pGL3 reporter vectors encoding firefly luciferase under the control of a sensitive hypoxia response element and *Renilla* luciferase as a control of transfection efficiency. Cells were placed in hypoxic conditions (1% O_2) for 4 h in order to accumulate endogenous HIF-α before being collected. Results are given in percentage of firefly luciferase activity normalized to *Renilla* luciferase activity. The amount of HA-PHD2 transfected (PHD2) was quantified by immunoblotting using an anti-HA antibody. **B.** The effect of PHD2 effect on HIF-2α protein stability in a one-hybrid reporter assay. Cells were co-transfected in a 6-well format with various amounts of pcDNA3-HA-PHD2 expression vectors (200, 100 and 50 ng), a Gal4-VP16-HIF-2αODD (amino acids 404-569) construct as well as a Gal4 response element-driven firefly luciferase reporter and a *Renilla* luciferase control plasmid. Twenty-four h post-transfection cells were incubated for 16 h in normoxic or hypoxic conditions. Results are mean values of three independent experiments performed in triplicate.

Figure 3

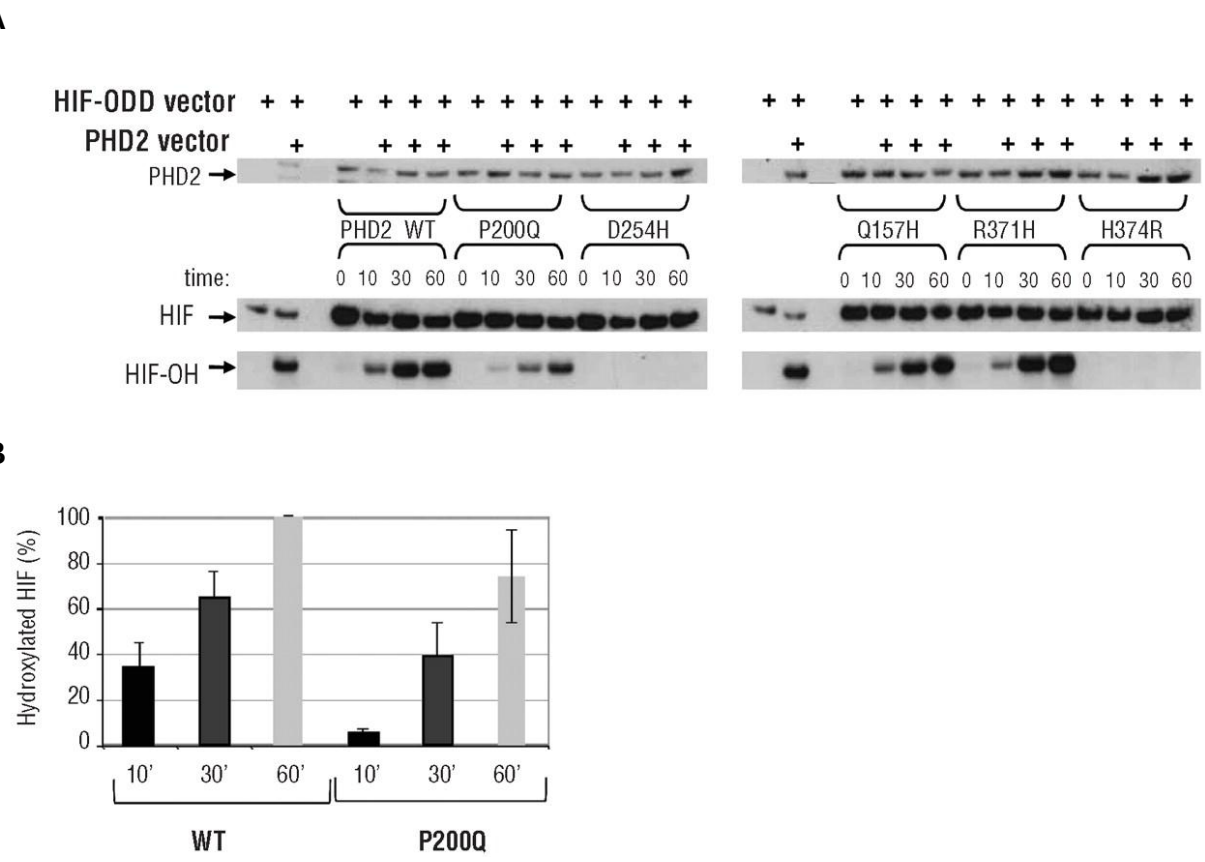


Figure 3 Functional analysis of *PHD2* mutants using an in vitro hydroxylation assay. **A.** Immunoblot estimation of HIF-1α-ODD protein hydroxylation *in vitro*. PHD2 and HIF-1α-ODD proteins were synthesized separately by *in vitro* transcription-translation reactions. The hydroxylation reaction was then processed by mixing HA-PHD2 and HA-HIF-1α-ODD proteins in a reaction buffer containing PHD2 enzymatic co-factors. The reaction was carried out at 30°C and samples were collected after 0, 10, 30, and 60 min of incubation. For immunoblotting, 10 µL aliquots of the hydroxylation reaction assays were separated by SDS-PAGE, blotted, and incubated with an anti-HA antibody [to quantify PHD2 and total HIF-ODD (HIF)] and anti-hydroxylated HIF-1 antibody [to quantify hydroxylated HIF-ODD (HIF-OH)]. **B.** Quantification of hydroxylated HIF-1α. The proportion of hydroxylated HIF-1α was measured and related to total HIF-1α. The 100% value corresponds to the quantity of HIF-1α hydroxylated by the PHD2-WT protein after 1 h. Means were obtained with three independent experiments.

Unpublished data

Since the HIF transcriptional and stability reporter assays (figure 2A and B) were performed in Hek293T and HeLa cells, which do not express Epo, we repeated both experiments with the P200Q-PHD2 mutant in an Epo-expressing system (Hep3B cells, see figure 4). However, no significant differences could be observed between mutant and wild type PHD2. Further experiments are required in order to elucidate the functional meaning of this mutation on the PHD/VHL/HIF pathway. In particular, a PHD2-deficient background would be necessary in order to avoid interference of the endogenous protein with the assays. Additionally, repeating the experiments in a kidney-derived, more physiological cell model would be helpful to understand the real effect of the P200Q mutation on PHD2 functionality. However, such renal cell model is currently not available (see next chapter).

Figure 4

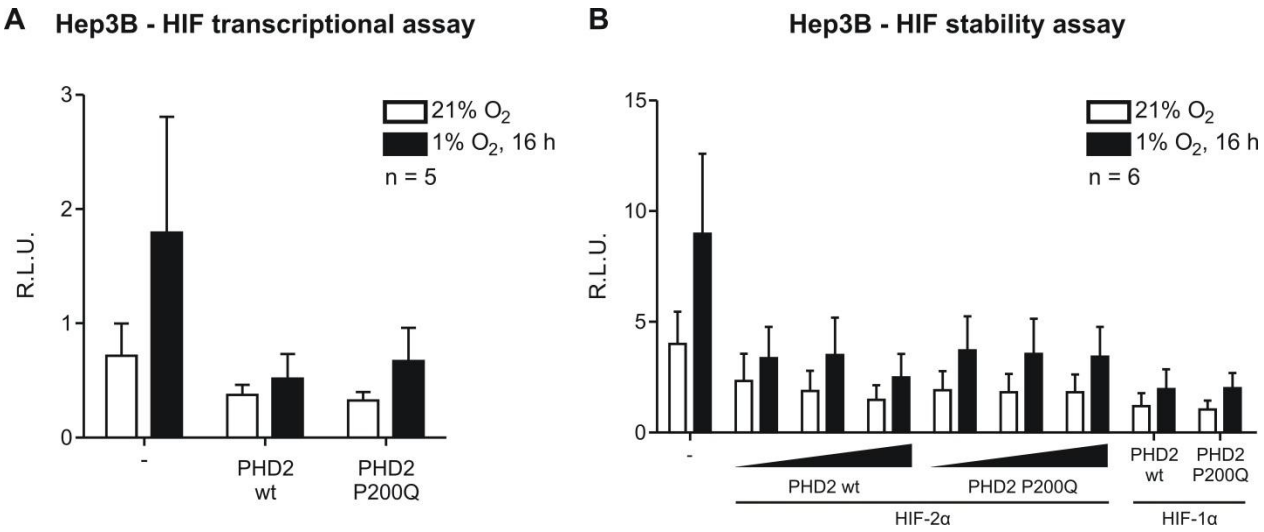


Figure 4 HIF transcriptional (**A**) and stability (**B**) assays were performed in Hep3B cells transfected with the wt and P200Q mutant PHD2-expressing vectors, as in figure 2. The stability assay was performed with both HIF-1α and HIF-2α ODD and with increasing amount of the PHD2-expressing constructs (50, 100 and 200 ng). Results are mean values of 3 independent experiments performed in triplicates and shown as ratio of firefly to *Renilla* relative luciferase units (R.L.U.).

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5. Epo-producing cellular models

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Introduction

In order to study erythropoietin (Epo) hypoxia-regulated expression *in vitro*, we first characterize a series of cellular models for their ability to produce Epo in response to reduced oxygen tension or hypoxia-like stimuli (hypoxia mimetic compounds). Historically, the hepatocellular carcinoma-derived human cell lines HepG2 and Hep3B have been used as models for Epo oxygen-dependent expression (Goldberg et al., 1987; Warnecke et al., 2004). Recently, a novel renal cell model capable of hypoxia-induced Epo production was reported and showed for the first time several features typical of the renal Epo-producing cells (REPCs) *in vivo* (Frede et al., 2011). REPCs were isolated form the tumour-free tissue of a patient with renal carcinoma and have been shown to express 5'-ectonucleotidase (CD73), microtubule-associated protein 2 (MAP2) and neurofilament light polypeptide (NFL), known markers overlapping with Epo expression in kidney cells. Moreover, REPCs displayed dendritic-like processes and the typical negative feedback regulation of Epo mRNA during chronic hypoxia, with a peak after 48 h at 1% O_2. We obtained and extensively characterized this model but, unfortunately, DNA profiling using 8 different and highly polymorphic short tandem repeat (STR) loci revealed that REPCs were cross-contaminated with Hep3B. The same analysis was independently performed by several groups that received different batches of REPCs, including our group, all confirming the hepatic identity of what were thought to be REPCs. Consequently, the paper describing REPCs by Frede et al. (Frede et al., 2011) was retracted from the journal *Blood* (see below). Besides kidney and liver, the brain represents the third organ with relatively abundant Epo expression. It's not surprising that two neuroblastoma cell lines, Kelly and SH-SY5Y, have been shown to express Epo mRNA in an oxygen-dependent way (Stolze et al., 2002).

Materials and methods

Cell culture and reagents

Hep3B, HepG2, REPCs and Hek293T were cultured in DMEM (high glucose, Sigma-Aldrich) supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Kelly and SH-SY5Y cells were cultured in RPMI (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Hypoxia experiments were carried out at the indicated concentration of oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies). Dimethyloxaloylglycine (DMOG, D1070, Frontier Scientific) was dissolved in dimethyl sulfoxide (DMSO) and used at the concentration of 1 mM for 8 h. Deferoxamine (DFX, Sigma-Aldrich) was dissolved in water and used at a concentration of 100 µM for 16 h.

RNA extraction and quantitative PCR

RNA was extracted by using the phenol-chloroform method and cDNA was generated by reverse transcription (RT) of 2 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were quantified by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma-Aldrich) in combination with a MX3000P light cycler (Agilent) and the primers listed in table 1. Initial template concentrations were calculated by comparison with serial dilutions of a calibrated standard. Ribosomal protein L28 mRNA levels were used to normalize the data.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size
hL28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198 bp
hEpo	TCACTGTCCCAGACACCAAA	CCTCCCCTGTGTACAGCTTC	362 bp
hCAIX	GGGTGTCATCTGGACTGTGTT	CTTCTGTGCTGCCTTCTCATC	309 bp
hHIF-1α	CATAAAGTCTGCAACATGGAAGGT	ATTTGATGGGTGAGGAATGGGTT	148 bp
hHIF-2α	TTGATGTGGAAACGGATGAA	GGAACCTGCTCTTGCTGTTC	196 bp
hPAI-1	ACTGGAAAGGCAACATGACC	GAGGAAGGGTCTGTCCATGA	296 bp

Table 1 Primers used for qPCR amplification

HIF-α knockdown cells and lentiviral transduction

Lentiviral expression vectors encoding shRNA sequences targeting human HIF1A at nucleotides 1168-1188 (NM_001530.x-1048s1c1), human HIF2A at nucleotides 2055-2075 (NM_001430.x-1694s1c1) and a non-targeting control sequence in a pLKO.1-puro plasmid were purchased

from Sigma. Viral particles were produced in Hek293T cells by co-transfection of the respective transfer vector (3 µg) with the packaging plasmids pLP1 (4.2 µg), pLP2 (2 µg) and pVSV-G (2.8 µg, all from Invitrogen) using PEI transfection as previously described (Stiehl et al., 2012). Cells were transduced with lentiviral-pseudotyped particles and cell pools were derived by puromycin selection.

Immunoblotting

Combined cytoplasmic and nuclear extracts were prepared using a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 x protease inhibitory cocktail (Sigma-Aldrich). Protein concentrations were determined by the bicinchoninic acid assay method and up to 80 µg of cellular protein was subjected to immunoblot analyses. Membranes were probed with antibodies against HIF-1α (clone 54/HIF-1α, BD Transduction Laboratories), HIF-2α (PAB12124, Abnova) and β-actin (Sigma-Aldrich). Signals from HRP-coupled secondary antibodies were detected with ECL substrate (all Pierce) using a luminescent image analyser (LAS-4000, FUJIFILM). β-actin was used as loading control.

Results

Hepatoma cell lines

HepG2 and Hep3B are known to lose Epo hypoxic inducibility over cell culture passages and often require sub-cloning in order to keep high Epo induction folds upon hypoxic exposure. We therefore tested different batches of cells available in the lab and chose the ones showing the most optimal hypoxia-inducibility in terms of Epo mRNA for further experiments (figure 1). We also used DMOG as a hypoxia mimetic and confirmed the expected increase in Epo transcription after 8 h of treatment. DMOG is an ester of N-oxalylglycine that penetrates cells readily and inhibits all prolyl-4-hydroxylase domains (PHDs) 1–3 and factor-inhibiting HIF (FIH). DMOG competes with PHD endogenous substrate, 2-oxoglutarate, and inhibits the hydroxylation reaction, resulting in hypoxia-inducible factor (HIF) stabilization (Jaakkola, Mole et al. 2001).

Renal Epo-producing cells (REPCs)

Before the STR loci analysis was performed and the paper describing REPCs was retracted, we extensively characterized the cell line. Results obtained with this model will be labelled

hereinafter with “exREPCs” and will be treated as from an alternative batch of Hep3B cells. ExREPCs showed Epo mRNA induction upon exposure to hypoxia, in a time- and oxygen-dependent manner, as well as to common hypoxia mimetics (figure 2). Importantly, we couldn’t recapitulate the feedback regulation in Epo transcription levels upon chronic hypoxia, as expected from *in vivo* and *in vitro* data (Jelkmann, 1982; Frede et al., 2011). In addition to DMOG, we used DFX, an iron-chelator. Iron is required for hydroxylation of HIF subunits by PHDs, thus DFX stabilizes HIFs by sequestering iron and inhibiting the hydroxylation reaction (Keberle, 1964).

Moreover, we generated stable HIF-1α and HIF-2α deficient cell lines by using a lentiviral-mediated knockdown approach to dissect the role of the single HIFα subunits in Epo hypoxic induction. As expected (Warnecke et al., 2004; Wenger and Hoogewijs, 2010), knockdown of HIF-2α, but not HIF-1α, resulted in significant reduction of Epo mRNA upon hypoxia when compared to control cells (figure 3). Efficiency of the knockdown was evaluated at the RNA and protein level, as well as by measuring mRNA levels of isoform-specific target genes, CAIX (carbonic anhydrase 9) for HIF-1α and PAI-1 (plasminogen activator inhibitor type 1) for HIF-2α. Increased HIF-2α protein levels were observed in HIF-1α deficient cells, followed by increased PAI-1, but not Epo, mRNA levels (figure 3B and C). This fact is not unexpected and has already been shown in different breast cancer cell lines (Fuady et al., 2014).

Neuroblastoma cell lines

Two cell lines derived from patients with neuroblastoma, Kelly and SH-SY5Y, were described in literature for their ability to produce Epo in an oxygen-dependent way (Stolze et al., 2002). We exposed them to hypoxia and measured Epo mRNA induction: Kelly cells displayed high Epo expression in hypoxia, whereas SH-SY5Y cells showed much lower levels of Epo mRNA and only a slight induction after 24 h of hypoxic exposure (figure 4).

Discussion

A prerequisite to investigate Epo regulation *in vitro* is a cell model with robust and reliable Epo hypoxic induction. Therefore, we tested several cell lines known to express oxygen-dependent Epo by exposing them to different % of O_2 or to hypoxia mimetics, such as DMOG and DFX. The hepatoma cell lines HepG2 and Hep3B showed the expected induction in Epo mRNA with different kinetics: HepG2 displayed higher absolute levels of Epo compared to Hep3B but lower

hypoxic induction folds, mainly at early time points (4-8 h). Epo mRNA in Hep3B remained high even after prolonged hypoxia (128 h exposure, figure 1A and B). Both hepatoma cell lines also responded to DMOG, an inhibitor of PHD enzymes, by increasing Epo expression (figure 1C and D). Hep3B were chosen for further experiments, as Epo hypoxic induction was remarkably stronger than in HepG2. Despite several batches of HepG2 were tested, Epo mRNA levels did not further increase in hypoxia (data not shown).

Even though we extensively characterized REPCs, this model turned out to be a peculiar sub-population of Hep3B: we thus used them as an alternative source of hepatoma cells. Indeed, exREPCs showed time- and oxygen-dependent induction of Epo mRNA, with stable Epo levels after prolonged exposure (up to 6 days). DMOG and DFX were used as hypoxia mimetics and were able to increase Epo expression (figure 2). HIF-2α specificity for the *EPO* gene was also confirmed by generation of stable knockdown cell lines for the two HIFα subunits (figure 3). It must be mentioned that the genes tested, including Epo, were not totally HIF-1 or HIF-2 specific, meaning that non-statistically significant reduction of mRNA levels occurred also in cells silenced for the other HIF-α subunit (figure 3B). PAI-1 on the other hand displayed increased mRNA levels in HIF-1α silenced cells, consistently with increased HIF-2α protein levels in these cells (figure 3B and C). The effect of HIF isoform knockdown is thus not clear-cut and seems to be gene-specific. The retraction letter for the paper describing REPCs as a novel, kidney-derived cell line (Frede et al., 2011) is reported below.

The neuroblastoma cell models Kelly and SH-SY5Y showed the ability to increase Epo transcription when exposed to hypoxia at different extents. Absolute Epo levels in SH-SY5Y were significantly lower than in Kelly, as well as the hypoxic induction folds (figure 4). We thus decided to proceed with Kelly cells as a non-hepatic, Epo-producing *in vitro* model.

A kidney-derived cell line capable of expressing Epo when exposed to hypoxia is still missing and would be the most appropriate model to study renal Epo regulation. Attempts were made to isolate primary REPCs but they all failed due to impossibility of keeping the cells in culture or loss of Epo expression over a short period of time. Our lab extensively tried to establish the mouse kidney-derived E4 line as an Epo *in vitro* model (Plotkin and Goligorsky, 2006), but the few Epo-expressing cells in the mixed population seem to be lost with culture passages even after subcloning (Sara Santambrogio, data not shown). It is possible instead to target Epo-producing cells *in vivo* and we are currently generating transgenic mice carrying the tamoxifen-dependent Cre recombinase (Cre^{ERT2}) under the control of the mouse *Epo* locus using the bacterial artificial chromosome (BAC) technology. Crossing the *Epo*-Cre animals with mice

baring different floxed alleles allows studying the role of the floxed gene of interest in renal Epo regulation (see next chapter).

Figure 1

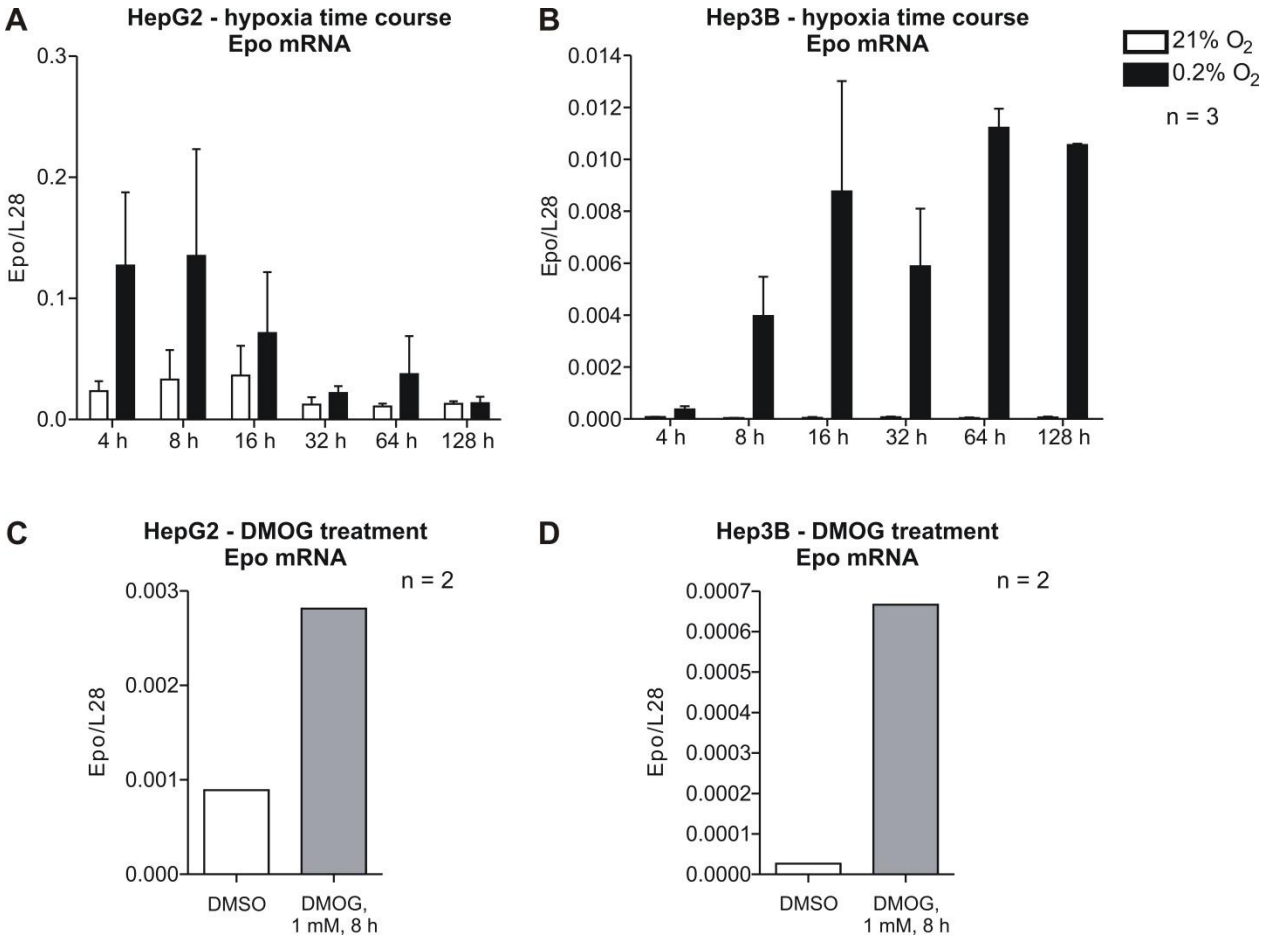

Figure 1 HepG2 (**A-C**) and Hep3B (**B-D**) were exposed to 0.2% O_2 or to 1 mM DMOG for the indicated time points and Epo mRNA levels were measured by RT-qPCR. Data are expressed as mean ± SEM or mean alone.

Figure 2

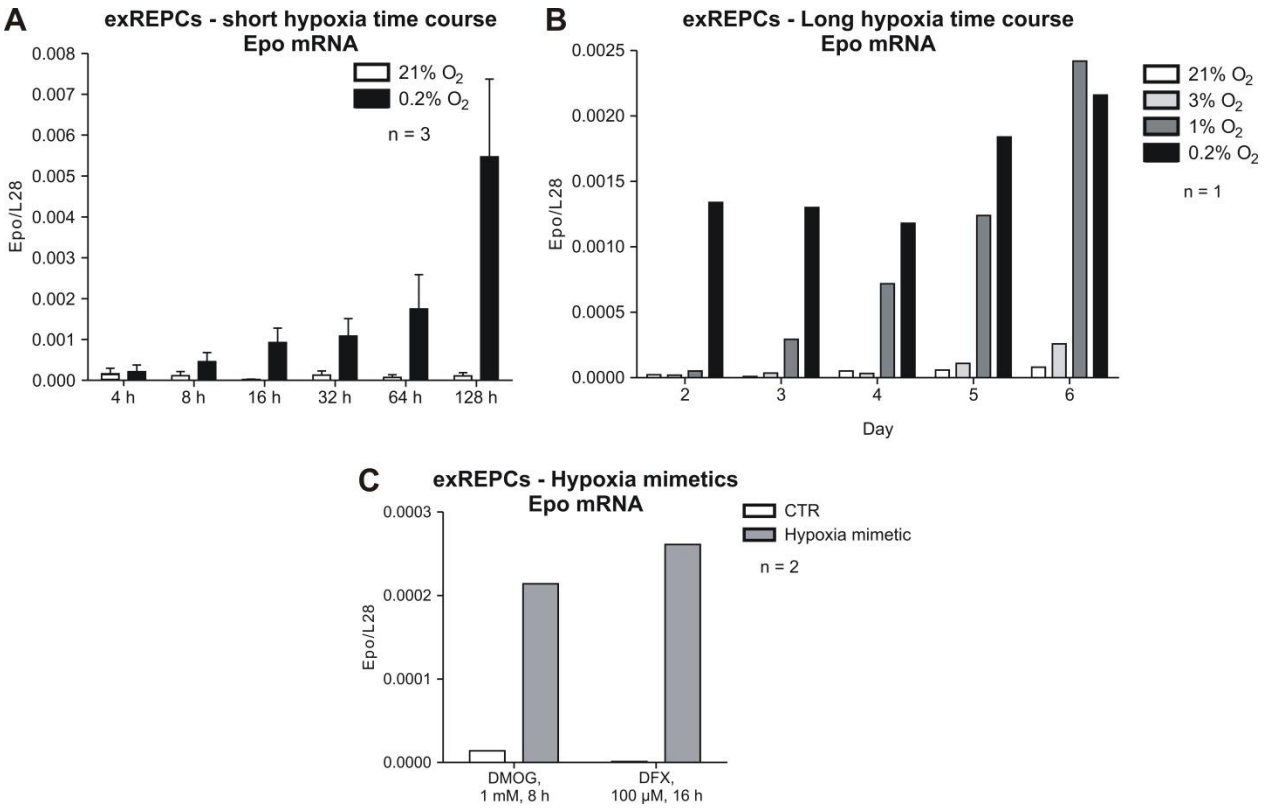

Figure 2 ExREPCs were exposed to different O_2 concentration (**A** and **B**) or hypoxia mimetics (**C**) for the indicated time points and Epo mRNA levels were measured by RT-qPCR. Data are expressed as mean ± SEM or mean alone.

Figure 3

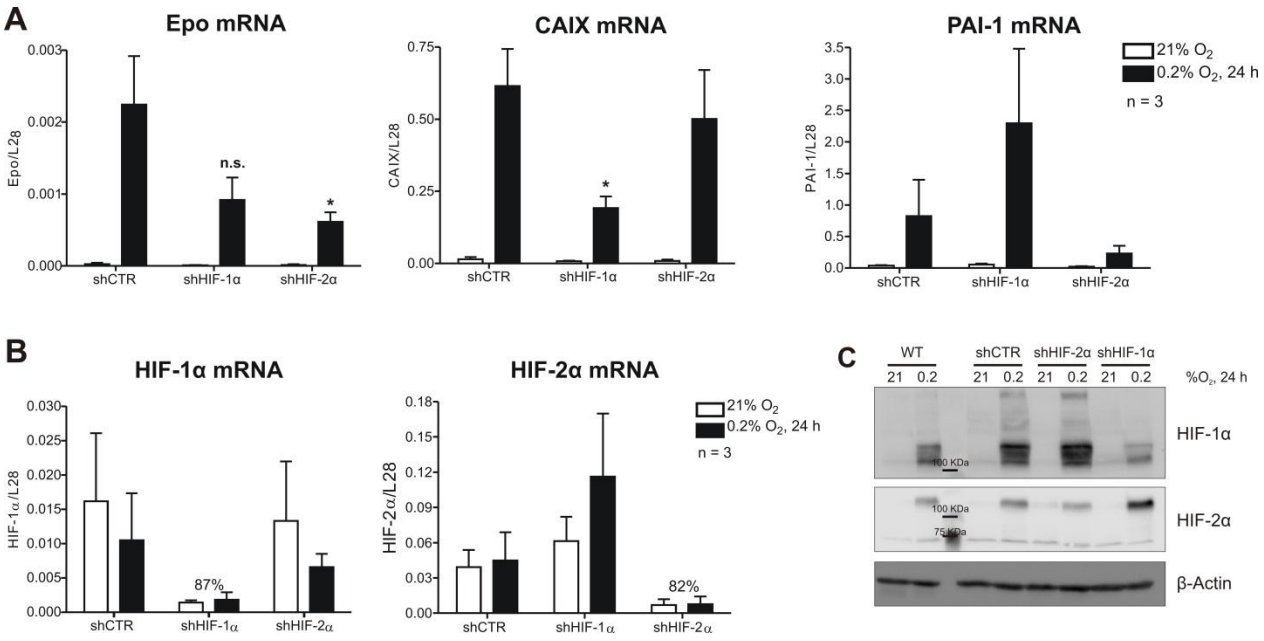

Figure 3 Stable HIF-1α and HIF-2α knockdown in exREPCs was generated by means of lentiviral transduction. Control (shCTR), HIF-1α silenced (shHIF-1α) and HIF-2α silenced (shHIF-2α) cells were exposed to 0.2% O_2 for 24 h and Epo, CAIX, PAI-1 (**A**), HIF-1α and HIF-2α (**B**) mRNA levels were measured by RT-qPCR. Efficiency of the knockdown is shown as % of shCTR cells and was evaluated at the protein level as well as by immunoblotting (**C**). Data are expressed as mean ± SEM. Statistics: student t-test, *p < 0.05, n.s. = not significant.

Figure 4

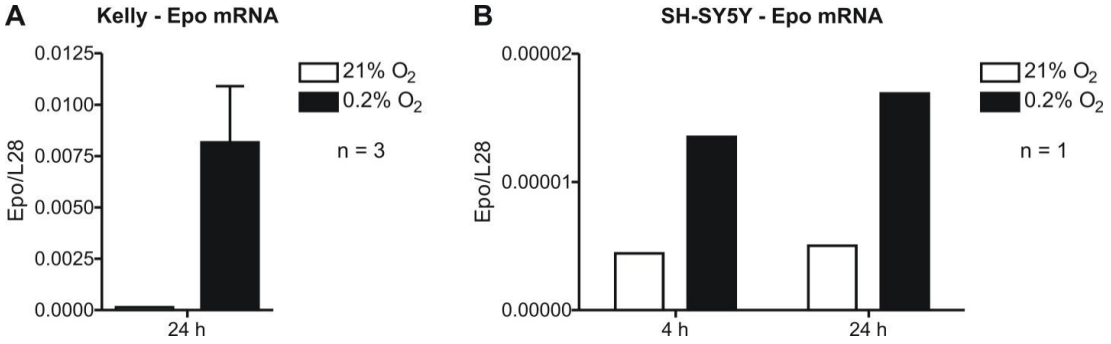

Figure 4 Kelly (**A**) and SH-SY5Y (**B**) were exposed to 0.2% O_2 for the indicated time points and Epo mRNA levels were measured by RT-qPCR. Data are expressed as mean ± SEM or mean alone.

Retraction letter

Frede S, Freitag P, Geuting L, Konietzny R, Fandrey J. Oxygen-regulated expression of the erythropoietin gene in the human renal cell line REPC. *Blood*. 2011;117(18):4905-4914.

The Editors of Blood wish to retract the above-mentioned publication.

The paper reported on a permanent human renal cell line, which was established from the tumor-free renal tissue of an anonymized male patient suffering from a kidney tumor. The cells, which were capable of hypoxia-inducible erythropoietin production and therefore termed renal erythropoietin producing cells (REPC), showed features of neuronal cells and were negative for hepatitis virus B.

After publication, the authors have freely provided the cells to a number of researchers who requested them. One of the collaborators has brought to the authors' attention that the REPC were contaminated with human Hep3B cells. Upon this, the authors have externally tested the cells including frozen aliquots of the youngest passage that were left after treating all REPC for mycoplasma infection. DNA profiling using 8 different and highly polymorphic short tandem repeat (STR) loci revealed that the REPC were cross-contaminated with Hep3B. STR profiles of the used cells matched the STR reference profile of the cell line Hep3B as indicated by a search of the STR database from the cell banks ATCC (United States), HPACC (United Kingdom), JCRB (Japan), RIKEN (Japan), KCLB (Korea) and DSMZ (Germany). Upon this, all groups who received cells were immediately informed about the contamination and have been asked to send back aliquots of the cells. These were again externally tested, but the contamination was confirmed. One collaborating group had identified different cell phenotypes in the REPC cultures by immunohistochemistry and tried to isolate single clones from the phenotypically different-appearing cells. The latest STR profiling of the 2 clones, however, also revealed persisting cross-contamination with Hep3B.

Thus, the authors notified Blood that they can currently neither provide cells that were described in the original publication nor reproduce the original results due to the lack of the cells. Because the cells should have served as an important new *in vitro* model or renal cells to study erythropoietin production, the authors and the Journal feel obliged to retract the above publication to avoid any further confusion about the existence of a permanent human renal

erythropoietin-producing cell line. The authors apologize for any inconvenience that the research community may have faced with REPC obtained directly from them or from other sources.

All authors agree to the retraction.

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Wenger RH, et al. (2010) Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. *Am J Physiol Renal Physiol* **298**(6): F1287-F1296.

6. Candidate factors involved in Epo regulation

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Introduction

In the last years, a number of proteins regulating the von Hippel-Lindau protein/prolyl-4-hydroxylase domain/hypoxia-inducible factor (pVhl/PHD/HIF) pathway have been identified, some of them by our group (Köditz et al., 2007; Balamurugan et al., 2009; Wenger et al., 2009; Wenger and Hoogewijs, 2010; Wollenick et al., 2012; Chiang et al., 2013; Pawlus et al., 2013). We aimed to evaluate the effect of selected established as well as novel interactors of the oxygen-sensing signalling on erythropoietin gene (*EPO*) transcription in hypoxia. After measuring endogenous expression of such factors in Epo-producing cells, we chose to silence the genes of interest by small interfering or short hairpin RNAs (si- or shRNAs) in order to generate transient or stable deficient cells, respectively, and evaluate the consequences of the knockdown on Epo mRNA levels. Moreover, we combined the knockdown approach with luciferase reporter assays using constructs containing the Epo 5' hypoxia-response element (HRE, see chapter 3) to investigate a potential effect/binding of the candidate factors on the novel regulatory element. When possible, overexpression of the gene of interest was used as a parallel strategy. A short overview of the analysed transcription factors and the rationale of our choice are provided in the results section.

Materials and methods

Cell culture and reagent

Hep3B, HepG2, exREPCs, Hek293T and PC-3 were cultured in DMEM (high glucose, Sigma-Aldrich) supplemented with 10% heat-inactivated foetal calf -serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Hypoxia experiments were carried out at the indicated concentration of oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies).

RNA extraction and quantitative PCR

RNA was extracted by using the phenol-chloroform method and cDNA was generated by reverse transcription (RT) of 2 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were quantified by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma-Aldrich) in combination with a MX3000P light cycler (Agilent) and the primers listed in table 1. Initial template concentrations were calculated by comparison with serial dilutions of a calibrated standard. Ribosomal protein L28 mRNA levels were used to normalize the data.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size
hL28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198 bp
hEpo	TCACTGTCCCAGACACCAAA	CCTCCCCTGTGTACAGCTTC	362 bp
hETV-4	ACGGACTTCGCCTACGACTCA	CCTGGCGACCTCCTCAGGCT	348 bp
hVIM	GAGAACTTTGCCGTTGAAGC	TCCAGCAGCTTCCTGTAGGT	170 bp
hCITED-2	GGAGCAGAAATCGCAAAAAC	GACCCATGAACTGGGAGTTG	334 bp
hCAIX	GGGTGTCATCTGGACTGTGTT	CTTCTGTGCTGCCTTCTCATC	309 bp
hHIF-1α	CATAAAGTCTGCAACATGGAAGGT	ATTTGATGGGTGAGGAATGGGTT	148 bp
hHIF-2α	TTGATGTGGAAACGGATGAA	GGAACCTGCTCTTGCTGTTC	196 bp
hUSF-2	AAAAATTGATGGAACCAGAACACC	TCCCGTCTTGCTGTTGTC	151 bp
hPAI-1	ACTGGAAAGGCAACATGACC	GAGGAAGGGTCTGTCCATGA	296 bp
hVEGFA	CTACCTCCACCATGCCAAGT	TGGTGATGTTGGACTCCTCA	263 bp
hATF-4	TCAAACCTCATGGGTTCTCC	GTGTCATCCAACGTGGTCAG	226 bp
hPAG-1	CTGATGAACGTGCCTTCAGA	CATTTTGGTTTCCCTGTGCT	215 bp
hDNMT3A	TATTGATGAGCGCACAAGAGAGC	GGGTGTTCCAGGGTAACATTGAG	111 bp

Table 1 Primers used for qPCR amplification

Generation of stable knockdown cell lines

Lentiviral expression vectors encoding the indicated shRNA sequences in a pLKO.1-puro plasmid were purchased from Sigma. Viral particles were produced in Hek293T cells by co-transfection of the respective transfer vector (3 µg) with the packaging plasmids pLP1 (4.2 µg), pLP2 (2 µg) and pVSV-G (2.8 µg, all from Invitrogen) using polyethylenimine (PEI) transfection as described before (Stiehl et al., 2012). Cells were transduced with lentiviral-pseudotyped particles and cell pools were derived by puromycin selection.

RNA interference

Hep3B cells were plated at a density of 4×10^5 cells per single well of a 6-well plate or of $1.5x10^6$ per 10 cm dish. The day after, cells were transfected in fresh media with 300 pmol of the indicated siRNA duplexes (Invitrogen/Dharmacon) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Cells were evenly split onto 24- or 6-well plates 24 h post transfection and exposed to 21% or 0.2% oxygen for an additional 24 h before analysis.

Human Epo ELISA

Hep3B cells were plated at a density of 4×10^5 cells per single well of a 6-well plate. The day after, medium was replaced with 1.5 mL of DMEM, containing 10% FCS and antibiotics and cells were exposed to 21% or 0.2% of oxygen for 8 or 24 h. Medium was collected on ice, centrifuged in order to remove cells in excess and stored at -80°C. Medium from an empty well (no cells) was used as a control for the experiment. Human Epo ELISA (human erythropoietin Quantikine IVD ELISA Kit, DEP000, R&D Systems) was performed according to the manufacturer's instruction.

Immunoblotting

Combined cytoplasmic and nuclear extracts were prepared using a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 x protease inhibitory cocktail (Sigma-Aldrich). Nuclear extracts were prepared using a low salt followed by a high salt extraction buffer containing 0.15 or 0.4 M NaCl, respectively, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 x protease inhibitory cocktail (Sigma-Aldrich). Protein concentrations were determined by the bicinchoninic acid assay method and up to 80 µg of cellular protein was subjected to immunoblot analyses. Membranes were probed with antibodies against HIF-1α (clone 54/HIF-1α, BD Transduction Laboratories),

HIF-2α (PAB12124, Abnova), ETV-4 (20580002, Novus Biologicals), USF-2 (N-18, SC-861; Santa Cruz Biotechnology), SP-1 (SC-59, Santa Cruz Biotechnology), ATF-4/CREB-2 (C-20, SC-200, Santa Cruz Biotechnology), PAG-1 (MAB0935, Abnova) and β-actin (Sigma-Aldrich). Signals from HRP-coupled secondary antibodies were detected with ECL substrate (all Pierce) using a luminescent image analyser (LAS-4000, FUJIFILM). β-actin was used as loading control for combined cytoplasmic and nuclear extracts, SP-1 was used as loading control for nuclear extracts.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed as described previously (Wollenick et al., 2012) using 5 µg of anti-USF-2 (C-20, SC-862, Santa Cruz Biotechnolgy) or anti-flotillin antibody (H-104, SC-25506, Santa Cruz Biotechnology) as a negative control. ChIP samples were analyzed by RT-qPCR using the primers listed in table 2, designed to detect occupancy of the specified regions. To determine USF-2 occupancy, the change in cycle threshold relative to input DNA was calculated and expressed as a percentage.

DNA region	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size
Epo 5'-HRE	GATGGAGCTGTGTCTCCCTG	AGTGGCAATGTGGAGGTCTG	206 bp
Downstream	GGTGCTTGGTCAGGAGTTGA	GACACGTTCTCGTCCAACCT	79 bp
Epo 3'-HRE	CAGCAGTGCAGCAGGTCCAGGTCC	CGAGAGGTCAGACAGGCTGTGTGAG	89 bp
PAI-1 -248/-128	GGCTTTTTGGGTCACCCGGCA	ACCCCAATAGCCTTGGCCTGA	117 bp

Table 2 Primers used for ChIP analysis

Plasmids and luciferase assays

The following overexpressing plasmids were used in combination with luciferase reporter vectors described in chapter 3: pcDNA3.1hygro2xFLAG-hUSF-1/2 (Invitrogen, (Pawlus et al., 2012)), pcDNA3.1nV5-hATF-4 (Invitrogen, (Köditz et al., 2007)) and pcDNA3.1-hGATA-2/-3/-4 (Invitrogen, kindly provided by Chistof Dame, Charité-Universitätsmedizin Berlin, Germany). For reporter assays, $4x10^5$ Hep3B cells were transiently transfected with 500 ng firefly expressing plasmid and 500 ng of overexpressing vector in a six-well format by PEI (polyethylenimine). To control for differences in transfection efficiency and extract preparation, 5 ng pRL-SV40, pRL-CMV or pRL-TK *Renilla* luciferase reporter vectors (Promega) were co-transfected. Cultures were evenly split onto 24-well plates 24 h post transfection and exposed to 21% or 0.2% oxygen

for an additional 24 h. Luciferase activities of triplicate wells were determined using the Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Reporter activities were expressed as relative firefly/*Renilla* luciferase activities. All reporter gene assays were done at least 3 independent times. pH3SVL, PAI-1 and CAIX reporter constructs were previously described (Wanner et al., 2000; Pawlus et al., 2013).

Results

ETV-4

The E-twenty six transcription factor ETS translocation variant 4 (ETV-4), also known as polyomavirus enhancer activator 3 (PEA3), belongs to the ETS family of transcription factors. These factors are characterized by the ETS domain, capable of binding to DNA sequences with a 5′-GGA(A/T)-3′ core, even though factor-specific differences in preferential binding sites have been identified (Wei et al., 2010; Oh et al., 2012). ETV-1 and -5 are the closest relatives to ETV-4, thus belonging to the same sub-family, and overlapping physiological roles have been demonstrated at least for ETV-4 and -5. Branching morphogenesis, where primitive epithelial buds bifurcate to generate arborized ducts or acinar structures during embryogenesis, is certainly a process in which ETVs play a role (Chotteau-Lelievre et al., 2003). ETV-4 and -5 knockout mice failed to develop kidneys, while inactivation of single genes had no impact on nephrogenesis, suggesting that either gene can compensate for the other one in the context of kidney organogenesis (Lu et al., 2009). The main signalling pathways involved in kidney development include the glial cell derived neurotrophic factor/ret proto-oncogene (GDNF/Ret) and the fibroblast growth factor 10 (FGF-10) axes which, upon activation, lead to ETV-4 and -5 expression and activation (Costantini, 2010). Additionally, ETV-4 and -5 deficient animals showed defects in the reproductive function and both females and males were less fertile than control mice or even sterile, suggesting a role for ETVs in the reproductive system (Laing et al., 2000; Eo et al., 2011). Erythroid parameters, such as RBC count or Epo plasma levels, have not been extensively studied in these animals. Well established is instead the oncogenic potential of ETVs in different cancer types, where they are often overexpressed and correlate with adverse outcome. Factors of the PEA3 family can function as oncogenes in breast cancer, prostate cancer and Ewing's sarcoma, in which chromosomal translocations lead to formation of fusion proteins between the Ewing's sarcoma (EWS) gene and ETV-1 or -4. Moreover, the majority of prostate tumours display chromosomal translocations involving transmembrane protease serine

2 (TMPRSS2) and ETVs (Oh et al., 2012). ETVs can be regulated post-translationally by the E3 ubiquitin ligase COP-1, which recognises specific amino acid binding motives and targets ETVs for degradation. Consistently, COP-1 has been shown to behave as a tumour suppressor gene in prostate cancer (Vitari et al., 2011).

ETV-4 has been recently found to act as a broad co-activator of the HIF signalling. It contributed, among others, to *PHD2* gene expression under hypoxic conditions and was able to form complexes with HIF-1α and -2α (Wollenick et al., 2012). This observation, together with the importance of ETV-4 expression in the kidney, the main source of Epo, prompted us to investigate the possible involvement of ETV-4 in Epo hypoxic regulation.

We started by generating stable ETV-4 silenced cell lines by means of lentiviral transduction in exREPCs. The shRNA sequence used was remarkably efficient, both at the mRNA and protein levels (figure 1D, E and F), and analysis of vimentin (VIM) transcript, a known transcriptional target of ETV-4, confirmed functional silencing of the gene (figure 1C). Interestingly, Epo mRNA and protein levels were dramatically increased, both in normoxia and hypoxia in ETV-4 knockdown compared to control cells (figure 1A and B). Hypoxic induction fold of Epo mRNA was not significantly different between the two cell lines (data not shown).

Given the profound effect of ETV-4 knockdown on Epo mRNA and protein, we performed a more in-depth analysis of HIF-2, the main regulator of Epo transcription.We measured HIF-1α and -2α protein levels in normoxic and hypoxic cells and we could observe a significant and specific increase in HIF-2α levels in hypoxic ETV-4 deficient cells compared to control cells (figure 2A). Consistently, HIF-2 target gene Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED-2) mRNA levels showed a trend of induction in ETV-4 knockdown exREPCs, whereas HIF-1 target gene carbonic anhydrase IX (CAIX) levels were similar to control cells (figure 2B). Taken together, these data indicate that knockdown of ETV-4 in exREPCs results in increased Epo levels both in normoxia and hypoxia and that this effect could be mediated by increased HIF-2α levels, at least in hypoxia. Knockdown of HIF-2α would be necessary to prove its involvement in Epo regulation by ETV-4. The effect on HIF-2α would however not explain the increase in Epo levels observed in normoxic conditions.

The observations described above are based on a single sequence of shRNA directed against ETV-4 mRNA to generate stably silenced cell lines. To rule out shRNA off-target effects (Ramji et al., 2013; Fellmann and Lowe, 2014), we decided to knock ETV-4 down by using transient siRNA transfection or different shRNAs sequences in order to confirm its regulatory effect on

Epo. As shown in figure 3A, the strong increase in Epo mRNA observed in ETV-4 silenced cells was unfortunately not reproducible in exREPCs transfected with two different sets of siETV-4, despite very good knockdown efficiency. Two of the three siRNAs used showed a trend of increased Epo mRNA compared to the control siRNA (siETV-4 #1 and 3), but the effect was neither significant nor as dramatic as in the first round of stable knockdown (figure 1A). Moreover, a new series of ETV-4 deficient cells was generated with three different shRNAs targeting ETV-4, among which an identical sequence used in the first round (shETV-4 #33). Again, a slight increase in Epo mRNA could be observed with the most efficient shRNA (shETV-4 #33), but the trend was not present with the other sequences used (figure 3B). To avoid potential unspecific effects due to the shCTR sequence, a shRNA targeting GFP was used in this experiment as a control (shGFP).

Further complications came from the difficulties in measuring ETV-4 at the protein level. Different antibodies were tested for immunoblotting and, even if a band was recognized at the expected molecular weight (figure 1E), a pattern of multiple bands made it difficult to state which one really corresponded to ETV-4 protein. It must be mentioned that ETV-4 protein levels in exREPCs appeared to be lower compared to other cell lines, such as HepG2 or PC-3 (figure 1E). Thus, we decided to test the effect of ETV-4 knockdown on Epo in cell lines with higher expression of the gene of interest, such as HepG2. However, stably ETV-4 deficient HepG2 cells did not show differences in Epo mRNA levels neither in normoxia nor in hypoxia compared to control cells (figure 4).

In conclusion, the initial, pronounced effect of ETV-4 silencing on Epo mRNA levels observed in exREPCs could not be reproduced using independent methods or cell lines. Since the actual ETV-4 expression in renal Epo-producing cells *in vivo* is unknown, at the moment we cannot hypothesize an involvement of ETV-4 transcription factor in Epo regulation, despite its ability to regulate the oxygen-sensing pathway and interact with HIF-2α, at least in PC-3 cells (Wollenick et al., 2012).

USF-2

The second candidate factor we selected as a putative Epo regulator consists in the upstream stimulatory factor 2 (USF-2). USF-2 and its homolog USF-1, encoded by separate genes, are basic helix-loop-helix leucine-zipper transcription factors assembling in homo- or hetero-dimers to bind the E-box (CANNTG, where NN can be either GC or CG) core DNA sequence (Sirito et al., 1992). Non-canonical E-boxes or pyridine (Py)-rich initiator (Inr) sites can be bound by USFs

as well (Pawlus et al., 2012). The two isoforms mainly differ in the N-terminal activation domain, thus USF-1 and -2 can establish interactions with different transcriptional partners and have different impact on gene transcription. The E-box site can be bound by other transcription factors, such as the proto-oncogene c-Myc, well known to be involved in cell proliferation. USFs are ubiquitously expressed, albeit with tissue-specific differences, and the most abundant dimer in cell lines is usually represented by the USF-1/USF-2 heterodimer (Sirito et al., 1994). Hints on the physiological role of USF-2 come from animal experiments: USF-2 knockout mice showed clear growth defects after birth, with only 20% of the animals surviving this “critical period”, and lower body weight throughout adulthood (Vallet et al., 1997; Sirito et al., 1998). Interestingly, USF-2 expression was increased in USF-1-mutant mice (but not *vice versa*), suggesting that USF-2 can compensate for USF-1 loss, and an embryonic lethal phenotype was observed in double knockout mice, indicating that USFs are essential during development (Sirito et al., 1998). Glucose metabolism was also impaired in USF-$2^{-/-}$ mice, which showed decreased expression of liver enzymes involved in glucose response (Vallet et al., 1997). The link with glucose is additionally suggested by increased USF-1 and -2 expression upon glucose treatment *in vitro* and *in vivo* (Kahn, 1997; van Deursen et al., 2008). Higher levels of USFs activate in turn transcription of genes involved in extracellular matrix deposition and consequent fibrosis. Indeed, USF-2 transgenic mice showed worse outcome when diabetic nephropathy was induced (Wang et al., 2004; Liu et al., 2007), as well as increased activation of the renin-angiotensin system, also contributing to the pathologic phenotype (Shi et al., 2009). In addition, iron overload is often observed in USF-2-deficient animals but likely due to disruption of the USF-2 genomic locus which is in close proximity to the hepcidin gene, encoding for the major iron-regulatory peptide, and resulting in silencing of hepcidin. Erythroid parameters were analyzed only in the latter USF-$2^{-/-}$ mouse model mentioned and did not show alterations in adult USF-2 mutant mice compared to WT (Nicolas et al., 2001).

Our hypothesis of USF-2 as an Epo regulator relies on the cross-talk between USF and HIF pathways, which has been recently demonstrated *in vitro*. Recruitment of USFs to hypoxia-induced gene promoters affected transcription in different cancer cell lines in opposite ways, depending on the arrangement of the binding sites. When HREs and E-boxes overlapped, competition between USF and HIF dimers occurred, with subsequent inhibition of the HRE-dependent transcription. On the other hand, when the binding sites were close but not overlapping, mutual complementing effects were observed (Hu et al., 2011). Two reports in literature investigated the effect of USF-2 on HIF-2α specific target genes, including *EPO*. Knockdown of USF-2 significantly decreased hypoxic induction of several HIF-2, but not HIF-1,

target genes in Hep3B and USF-2 binding was detected on promoters and enhancer region of *EPO* and plasminogen activator inhibitor type 1 (*PAI1*) genes. USF-2 and HIF-2α were found to directly interact at the protein level and to recruit general activators of transcription, such as p300/CBP (Pawlus et al., 2012). Moreover, signal transducer and activator of transcription 3 (STAT3C) and USF-2 seem to be involved in HIF-target specificity: their binding to regulatory regions of HIF-1 and HIF-2 target genes, respectively, was demonstrated *in vitro*, as well as their ability to directly interact with the specific HIF-α subunit. Knockdown of STAT3C or USF-2 indeed resulted in impaired hypoxic inducibility of sets of HIF-1 or HIF-2 target genes (Pawlus et al., 2013). Based on these data and considering the fact that *EPO* is one of the most hypoxia-sensitive genes and a HIF-2 target, we aimed to study the effect of USF-2 silencing/overexpression on Epo mRNA and to assess its putative binding on the newly discovered 5'-HRE.

Transient USF-2 knockdown was generated in Hep3B cells by siRNA transfection, in parallel to HIF-2α knockdown as a positive control for blunted Epo hypoxic induction (figure 5). Two out of three siRNAs used resulted in efficient silencing of USF-2 at the mRNA level and, as expected, in reduced Epo transcript levels in hypoxia. CITED-2 mRNA was measured as an alternative HIF-2 target gene and displayed lower hypoxic induction in siHIF-2α and siUSF-2 #33 transfected cells. We concluded that USF-2 silencing was able to decrease Epo hypoxic induction, even though this effect was significant only with one of the siRNAs used against USF-2 mRNA and not as dramatic as in HIF-2α deficient cells or as previously reported (Pawlus et al., 2012).

In order to confirm the effect of USF-2 knockdown on Epo induction, we generated stably silenced Hep3B cells using two different shRNA sequences targeting USF-2. Figure 6 shows that efficient USF-2 knockdown (both at mRNA and protein levels) resulted in decreased amount of Epo mRNA upon hypoxic exposure of Hep3B. PAI-1 was used in this case as an alternative HIF-2 target gene and its transcript levels were indeed lower in USF-2 knockdown cells compared to control cells. It must be mentioned that PAI-1 levels differed a lot between WT and shCTR cells, a frequent observation in our hands.

Since binding of USF-2 on the Epo 3'-HRE has already been demonstrated in Hep3B (Pawlus et al., 2012), we performed *in silico* analysis of putative USF binding sites in the region surrounding the novel 5'-HRE using the JASPAR database web server with default settings (Mathelier et al., 2014) and found a consensus sequence for USF binding overlapping with the HIF binding site (HBS, figure 7). We next tested the activity of different Epo reporter constructs (described in

chapter 3) in USF-2 deficient cells, along with a PAI-1 promoter controlled vector as positive control (figure 8). USF-2 has previously been shown to contribute to PAI-1 hypoxic induction as well (Pawlus et al., 2013). In contrast to our expectations PAI-1 driven reporter activity was increased and not reduced in two different USF-2 silenced cell lines. Moreover, USF-2 knockdown seemed to have an effect on *Renilla* activity as well and, despite testing different promoters, increased *Renilla* counts were observed with all the constructs used (SV40-, CMV- and TK- promoter driven *Renilla*), thus making normalization of the assay not reliable (figure 8C). As no clear conclusion on the effect of USF-2 knockdown on Epo-reporter constructs could be drawn, we decided to switch to an overexpression system. The positive control PAI-1 reporter behaved as expected: increased firefly light units were measured when cells were co-transfected with USF-2, but not USF-1, overexpressing vector (figure 9). Nevertheless, the effect of USF-1 and -2 on the 5'-HRE-containing constructs was again not clear: consistently with previous data in USF-2 knockdown cells, decreased *Renilla* counts were observed in the USF-1/2 transfected samples compared to the control, thus precluding normalization of the assay. Moreover, we could not recapitulate the positive effect of USF-2 overexpression on the Epo promoter/3'-HRE reporter construct described by Pawlus et al. (Pawlus et al., 2013). The set of luciferase assays performed with USF-2 deficient or overexpressing cells was not sufficient to draw conclusions on the putative effect of USF-2 on Epo newly discovered 5'-HRE. Thus, we decided to investigate possible binding of USF-2 on the DNA region surrounding the HRE by ChIP. Again, PAI-1 and Epo 3'-HRE were used as positive controls and showed specific enrichment for USF-2 binding in hypoxia (figure 10), as expected from published data (Semenza and Wang, 1992; Yeo et al., 2008; Pawlus et al., 2013). However, no binding could be observed on Epo 5'-HRE, indicating that the USF-2 dependent Epo mRNA regulation seems to be mediated by the 3'- but not the 5'-HRE.

ATF-4

The activating transcription factor (ATF) family consists of a number of basic-leucine zipper transcription factors with broad functions and the ability to bind to a common DNA consensus sequence. The ATF binding site is identical to the later discovered cAMP responsive element (CRE), characterized by the core sequence 5'-ACGT-3' and bound by the CRE binding protein (CREB), one of the most well-known members of the ATF family (Deutsch et al., 1988; Lin and Green, 1989). Of note, ATF binding site can overlap with the HBS (5'-CGTG-3'), suggesting the possibility for a cross-talk with the HIF pathway. ATF-4 is a classical stress responsive gene: it is up-regulated at the transcriptional and translation level by different stress stimuli, including amino acid deprivation, endoplasmic reticulum (ER) stress, unfolded protein response (UPR),

oxidative stress and hypoxia. ATF-4 protein can interact with other members of the ATF family to form both homo- and hetero-dimers and act as a transcriptional activator or repressor, depending on the nature of the recruited co-factors (Ameri and Harris, 2008). ATF-4 is certainly known for its role in mediating the cellular response to ER stress: in condition of hypoxia or nutrient deprivation, unfolded or misfolded proteins accumulate in the ER and trigger the adaptive UPR, found already in yeast and conserved in mammalian cells (Alberts et al., 2002). The activation of the PKR-like endoplasmic reticulum kinase/eukaryotic translation initiation factor 2α (PERCK/eIF2α) pathway leads to AFT-4 stabilization and transcription of target genes, such as vascular endothelial growth factor (VEGF) and CCAAT/enhancer-binding protein homologous protein (CHOP), with the aim of ensuring amino acid availability for protein biosynthesis and protecting the cell form oxidative/hypoxic stress. When the cell cannot cope with the stress situation, UPR will ultimately result in apoptosis (Cullinan and Diehl, 2006).

Hypoxia can induce ER stress and UPR, thus resulting in increased ATF-4 mRNA and protein levels *in vitro* and *in vivo* (Blais et al., 2004; Rzymski et al., 2010; Tagliavacca et al., 2012). Primary rat fibroblasts have been shown to induce ATF-4 in anoxia (Estes et al., 1995) and ATF-4 levels were significantly higher in primary human tumours compared to corresponding normal tissues (Hiwatashi et al., 2011). More relevant to our aims, ATF-4 was identified as an interacting partner of PHD1 and PHD3, with subsequent changes in stability of the protein. In particular, PHD1 seemed to increase ATF-4 protein levels, while PHD3 had the opposite effect (Köditz et al., 2007; Hiwatashi et al., 2011). An oxygen-dependent degradation domain (ODDD) was additionally identified within ATF-4 amino acid sequence and a prolyl hydroxylation mechanism was proposed as the link between hypoxia and ATF-4 increased stability, similar to regulation of HIF-α subunits by oxygen (Köditz et al., 2007). The transcriptional effect of ATF-4 has been demonstrated for a few hypoxia-induced genes, including VEGF, CAIX and Epo itself. In conditions of hypoxia, activation of the PERCK/ATF-4 arm of the UPR pathway led to increased VEGF transcription and angiogenesis in different tumour models (Wang et al., 2012; Pereira et al., 2014). Moreover, ATF-4 directly interacted with HIF-1α in hypoxic osteoblasts *in vitro* and prevented its degradation, acting as a broad regulator of the HIF pathway (Zhu et al., 2013). The classical HIF-1 target gene CAIX can also be regulated by direct binding of ATF-4 on its promoter region, at least *in vitro*, and defects in ER stress response have been shown to impair CAIX transcription in hypoxia (van den Beucken et al., 2009). A recent report in literature investigated the effect of UPR on Epo transcription in HepG2 cells, both in basal conditions and after treatment with cobalt chloride, a hypoxia mimetic. Induction of ER stress led to increased ATF-4 levels, subsequent binding of the transcription factor to the Epo 3'-HRE and specific

inhibition of Epo transcription (Chiang et al., 2013). In addition to the *in vitro* data, ATF-4 knockout mice showed impaired development of the embryonic lens of the eye and severe foetal anaemia, persisting as mild anaemia in adult animals (Masuoka and Townes, 2002), suggesting again a link with Epo production, probably before birth. We therefore decided to investigate the effect of ATF-4 knockdown/overexpression in our Epo-expressing cell models and the possibility for ATF-4 to regulate Epo by binding to the 5'-HRE.

Generation of stably ATF-4 silenced Hep3B turned out to be problematic despite testing different shRNA sequences for knockdown efficiency. The highest silencing efficiency we could obtain was around 50% at the mRNA level and probably insufficient to detect effects on downstream target genes, such as VEGF and CAIX (figure 11). Detection of ATF-4 protein by immunoblotting proved to be equally challenging, due to antibody specificity issues. We observed no significant differences in Epo mRNA between control and shATF-4 cells but we could not exclude that the lack of any apparent regulation was due to inefficient silencing. We next screened for putative CREs in the region surrounding the Epo 5'-HRE in order to proceed with reporter assays combined with an overexpression approach. We made use of rVista, an online comparative genomics tool that aligns genomic DNA sequences of different species (human and mouse in this case) and searches for conservation of consensus transcription factor binding sites, implementing TRANSFAC database profiles (Loots et al., 2002). Indeed, rVista analysis showed a possible ATF-4 binding site shortly downstream of the HRE, with a spatial arrangement similar to Epo 3' enhancer (figure 12, (Chiang et al., 2013)). Based on the *in silico* data, we overexpressed ATF-4 or an empty control vector and evaluated the effect on different Epo reporter constructs, as well as on HIF-1 and HIF-2 specific reporters. None of the vectors used showed differences in terms of luciferase activity between control and ATF-4 overexpressing cells (figure 13). Importantly, CAIX reporter, used as a positive control (van den Beucken et al., 2009), was also not affected by ATF-4 overexpression. We therefore concluded that no effect of ATF-4 on Epo transcription can be observed in Hep3B cells neither in normoxia nor in hypoxia, at least under our experimental conditions.

GATA factors

As previously mentioned, GATA factors are established regulators of Epo transcription and tissue specific expression. The name of these transcription factors is derived from the DNA sequence of the binding site and up to now 6 members of the family have been identified (GATA-1 to -6) (Shimizu and Yamamoto, 2005). Known as master regulators of haematopoiesis, GATAs are zinc finger transcription factors expressed in a variety of tissues, including kidney

and liver. In normoxic conditions, GATA-2 and -3 have been shown to repress Epo transcription by binding to the GATA box present 30 bp upstream of the Epo transcriptional start site. Upon hypoxic exposure, GATA-2 and -3 binding markedly decreased along with dramatic Epo induction *in vivo* (Imagawa et al., 1997). GATA-2 and -3 expression was inversely correlated with Epo expression, the opposite of what has been observed for GATA-4, which followed Epo expression pattern in the post-natal switch from liver to kidney. Indeed GATA-4 was able to bind the same GATA box in the Epo minimal promoter and its knockdown led to impaired Epo transcription in hepatoma cell lines (Dame et al., 2004). The GATA binding site within the Epo promoter is fundamental to restrict Epo expression to specific cell types *in vivo*: a single nucleotide mutation in the GATA box resulted in constitutive ectopic Epo production by the renal distal tubule, the collecting duct and a certain population of epithelial cells in other tissues (Obara et al., 2008).

In order to evaluate putative binding of GATAs to the novel Epo 5'-HRE surrounding region, we applied the same approach used for the aforementioned transcription factors and started by performing an *in silico* analysis for predicted GATA boxes with the JASPAR database. As shown in figure 14, one such site was predicted in the region of interest; hence we overexpressed GATA-2, -3 and -4 together with the different Epo reporter constructs described in chapter 3 (figure 15). GATA-2 and -3 had an inhibitory effect on the Epo promoter-containing vectors, while GATA-4 showed the opposite trend, as expected. Unfortunately, addition of the 5'-HRE did not lead to significant changes in luciferase activity compared to the vectors containing the Epo promoter alone. As we only used the 100 bp (figure 15) and 300 bp (data not shown) long regions surrounding the HRE for this set of experiments, we cannot exclude that the GATA factors are able to bind outside of these fragments and regulate Epo transcription through the 5' kidney-inducible element as well.

DNMT3A

DNA methyl transferases (DNMTs) are a family of enzymes deputed to methylation at position 5 of cytosine within the CpG dinucleotide, an epigenetic marker of gene silencing (see introduction). Among this family, DNMT3A and 3B are responsible for *de novo* post-replicative DNA methylation but can also participate in maintenance of methylation pattern at heterochromatic, transcriptionally inactive regions (Okano et al., 1998). The expression of DNMT3A and 3B is high during embryogenesis and in undifferentiated cells and declines with development. Nevertheless, DNMT3A remains ubiquitously expressed at detectable levels (Jurkowska et al., 2011). DNMTs are essential for embryogenesis, as demonstrated by

embryonic or post-natal lethality of mice with inactivation of such genes (Li et al., 1992; Okano et al., 1999). Alterations in the genome methylation patter have deleterious consequences on gene expression and are thus often implicated in tumour progression: it is not surprising that mutations in DNMT3A have been identified in patients with acute myeloid leukemia (Ley et al., 2010; Yan et al., 2011) and associated to solid tumours (Kim et al., 2013). A recent publication by the group of Stephen Lee reported methylation and subsequent silencing of *EPAS1*, the gene encoding for HIF-2α, by DNMT3A in kidney primary cells and cell lines. Moreover, naturally occurring defects in DNMT3A were observed in kidney tumours, preventing silencing of HIF-2α and thus allowing the malignant cell to proliferate in hypoxic conditions (Lachance et al., 2014). We therefore aimed to modulate DNMT3A levels in Hep3B and evaluate a putative effect on HIF-2α and Epo expression.

After checking DNMT3A expression at the mRNA level in our cell model, we transiently knocked it down by siRNA transfection (figure 16). We used 3 different siRNAs, all efficiently targeting the gene of interest but obtained conflicting reulsts in terms of HIF-2α and Epo transcript levels. In particular, increased expression of HIF-2α upon DNMT3A knockdown, as expected from the work of Lachance and colleagues (Lachance et al., 2014), could not be recapitulated in our hands.

PAG-1

Phosphoprotein associated with glycosphingolipid-enriched mircodomains 1 (PAG-1), also known as C-terminal kinase (Csk)-binding protein (Cbp), is a transmembrane adapter protein broadly expressed in lipid rafts of mammalian cells. The main function of PAG-1 is to inhibit the ubiquitous Src pathway by binding to its main regulator, Csk (Brdička et al., 2000; Kawabuchi et al., 2000). Given the pro-proliferative, pro-angiogenic and ultimately transforming effect of Src pathway constitutive activation, it is not surprising that PAG-1 expression levels were associated with tumour progression due to its ability to regulate Src family kinases (Src, Fyn, Lyn, Yes, Hck, Lck, Blk, Fgr, Yrk). Depending on the origin of the tumour, PAG-1 has been proposed as an oncogene, like in renal cell carcinoma (Feng et al., 2009), or as a tumour-suppressor gene, like in colon cancer and non-small cell lung cancer (Oneyama et al., 2008; Kanou et al., 2011). Moreover, PAG-1 was recently identified as a hypoxia-induced gene with moderate but consistent induction of mRNA and protein levels *in vitro* and *in vivo* (Schörg et al., in preparation). Our interest in PAG-1 as a possible Epo regulator arises from studies investigating the stabilizing effect of Src kinases on HIF proteins. Src activation has been shown to inhibit hydroxylation of the HIF-1α subunit, thus leading to its stabilization even in normoxia in renal

carcinoma cell lines (Chan et al., 2002; Lee et al., 2011). The proposed mechanism is based on increased reactive oxygen species (ROS) production by Src, decreased vitamin C and subsequent PHD activity inhibition (Lee et al., 2011). VEGF transcription can also be downstream of Src pathway activation, since Src-deficiency or overexpression of dominant negative Src variants impaired VEGF induction in hypoxia (Mukhopadhyay et al., 1995). We therefore postulated a similar HIF-stabilizing effect by the PAG-1/Src axis followed by increased Epo synthesis and tested the hypothesis in our Epo-expressing cell models.

We confirmed hypoxic induction of PAG-1 mRNA in Hep3B exposed to different % of oxygen and for different time points (figure 17A). However, induction of PAG-1 at the protein level seemed to be much less pronounced compared to mRNA in this cell line, at least after 24 h at 0.2% O_2 (figure 17D: representative immunoblot). We efficiently knocked down PAG-1 with two different shRNA targeting sequences but could not see any significant changes in Epo mRNA levels; HIF-2α deficient cells were used alongside as a control for Epo regulation (figure 17B and C). Taken together, these data indicate no effect of PAG-1 silencing on Epo regulation in Hep3B cells under the experimental conditions used.

Discussion

In this study we investigated the role of several factors directly or indirectly involved in the PHD/HIF pathway on Epo regulation. Due to the lack of a more appropriate cell model the hepatoma cell line Hep3B was used for our studies. These cells are known to strongly induce Epo mRNA upon hypoxic exposure (Goldberg et al., 1987) and we could nicely reproduce Epo induction in our hands too (see chapter 5). As already mentioned, our main focus is Epo regulation in the kidney but culturing of renal Epo-producing cells (REPCs) has been so far unsuccessful. Thus, we used a liver-derived cell model, which is likely to have a different expression profile compared to kidney cells and does not fully recapitulate what can be observed in REPCs *in vivo*. Indeed, *in vivo* data from REPCs regarding the expression of the studied factors are lacking and would be useful for optimal choice of relevant candidates.

ETV-4 is expressed in whole mouse kidney *in vivo* and was recently shown to act as a broad co-activator of the HIF pathway (Wollenick et al., 2012). Since its interaction with HIF-2α, the main Epo regulator, was demonstrated *in vitro*, we investigated a potential ETV-4 effect on Epo transcription. Our experiments in Hep3B cells revealed that ETV-4 has no significant or reproducible effect on Epo regulation (figure 3). ETV-4 expression levels in Hep3B are not as

high as in other cell lines, such as PC-3 (figure 1E), again suggesting that the cell model used could not be optimal to study a putative ETV-4 dependent regulation effect on Epo transcription. We evaluated Epo expression in PC-3 and other abundant ETV-4 expressing cell lines but, as expected, we could not detect any Epo mRNA product after exposure to hypoxia (data not shown), making these cells not suitable to study Epo regulation. On the other hand, HepG2 cells showed higher levels of ETV-4 protein (figure 1E) and knockdown of ETV-4 in these cells did not result in significant changes in Epo mRNA levels (figure 4). Moreover, we could not show the general effect of ETV-4 knockdown on HIF-target genes that was previously observed in PC-3 cells (Wollenick et al., 2012). Expression levels of broadly hypoxia-inducible genes including CAIX, PHD2 and PHD3 were for instance unchanged between control and ETV-4 silenced cells (data not shown). ETV-5 is the second most important member of the PEA3 family of transcription factors and can compensate in certain cases for ETV-4 loss, as demonstrated by single mutant mice generation (Lu et al., 2009). However, ETV-5 expression was not significantly increased in ETV-4 knockdown cells, at least at the mRNA level (data not shown). It is likely that simultaneous knockdown of both members of the PEA3 family, ETV-4 and -5, is necessary to observe any effect on HIF target genes, including Epo.

USF-2 is also expressed in the kidney *in vivo* and its effect as a general co-activator of HIF-2 target genes has been demonstrated in the very same cell model we used, namely Hep3B (Pawlus et al., 2013). In particular, binding of USF-2 on Epo regulatory regions (minimal promoter and 3' enhancer) was observed upon hypoxic exposure and the factor was co-immunoprecipitated with HIF-2α subunit. The goal of this sub-project was to assess possible binding of USF-2 on the 5'-HRE surrounding region as well. We first confirmed reduced Epo hypoxic induction in USF-2 silenced cells via two independent approaches (transient and stable knockdown of the gene, figures 5 and 6), even though the effect was not as dramatic as previously shown (Pawlus et al., 2012) and not significant for the other HIF-2 target genes tested, CITED-2 and PAI-1. We continued with a series of reporter assays combining knockdown and overexpression of USF-2 with different Epo reporter constructs (see chapter 3). Unfortunately, luciferase assays gave unclear results due to variation in the activity of the normalizing *Renilla* gene and sometimes due to the controversial results of the control reporter vector (PAI-1 promoter driven firefly gene, figures 8 and 9). Finally, we performed ChIP experiments with an anti-USF-2 antibody but could not observe any enrichment in USF-2 occupancy of the 5'-HRE region. The fragment taken in consideration for the reporter and ChIP assays is 290 bp long, corresponding to the most conserved region around the 5'-HRE: we cannot rule out the possibility that the USF-2 binding site is further away from the HRE.

Nevertheless, based on these data the USF-2 dependent Epo regulation seems to be entirely mediated by regulatory regions other than the 5'-HRE.

The interaction of ATF-4 with the PHD/HIF pathway has been demonstrated *in vitro* and the effect of ER-stress response pathways, with subsequent ATF-4 activation, was shown in Hep3B to downregulate Epo hypoxic expression (Köditz et al., 2007; Hiwatashi et al., 2011; Chiang et al., 2013). The main problems encountered in this part of our study consisted in the low degree of knockdown achieved by shRNA transduction and in ATF-4 protein detection issues. Based on our results we cannot conclusively state whether silencing of ATF-4 has a significant impact on Epo mRNA levels. Luciferase assays showed no effect of ATF-4 overexpression on Epo reporters (figure 13); it must be mentioned however that CAIX promoter driven reporter construct, used as a control, also did not show the expected results (van den Beucken et al., 2009), making the interpretation of this set of experiment inconclusive. The above cited reports studying ATF-4 and HIF pathway interplay made use of compounds such as tunicamycin and thapsigargin to activate UPR and stabilize ATF-4 levels. Hence, another way to investigate ATF-4 dependent Epo regulation could be to treat Hep3B cells with the same compounds and activate a signalling cascade resulting in increased ATF-4 levels.

The link between the GATA factors and Epo regulation and the essential role of such factors for Epo tissue specificity are well established both *in vitro* and *in vivo* (Imagawa et al., 1997; Dame et al., 2004; Obara et al., 2008). A series of evidences speak for the novel Epo 5'-HRE as a region contributing to kidney-specific Epo expression, in a similar way to the well characterized 3' liver-inducible element. Animal experiments demonstrated that it is located within the putative kidney-inducible element, it contains a functional HRE, endogenous HIF-2α specifically binds to the HRE in hypoxic Hep3B cells and the surrounding region is extremely conserved in mammalian species (see chapter 3). Thus, we investigated binding of the GATA factors to the putative kidney inducible element by overexpressing GATA-2, -3 and -4 in our Epo reporter system. As expected, GATA-2 and -3 showed inhibition of reporter activity at different extent, with GATA-3 being the most effective factor; on the other hand, GATA-4 displayed an increase in luciferase activity. Addition of the 5'-HRE did not lead to significant changes compared to the vectors containing Epo minimal promoter and the 3'-HRE (figure 15). Once again, interpretation of the results is difficult due to variation in the *Renilla* reporter gene. Knock down of single GATA factors and ChIP experiments are required in order to draw conclusions about the effect and binding of the GATAs on the novel regulatory region. Moreover, GATA expression level in hepatoma cells is relatively high: an additional strategy consists in repeating the panel of

overexpression experiments in cells with low endogenous GATA factor expression, in order to avoid any background effect.

In regards to DNMT3A as a regulator of the HIF pathway, we did not obtain promising results by transient knockdown in Hep3B cells (figure 16). Since DNMT3A acts after replication of the cell DNA in order to maintain the methylation pattern throughout progeny, a possible explanation could be that our time window was too short and did not allow cells to replicate or to have a detectable effect on mathylation. A deeper analysis of *EPAS1* promoter methylation status, for instance via bisulfite treatment followed by DNA sequencing, would be necessary to set up the best experimental conditions.

We could instead conclusively show that silencing of PAG-1 did not influence Epo mRNA levels, neither in normoxia nor in hypoxia in hepatoma cells (figure 17). PAG-1 is a negative regulator of the Src family of kinases (Brdička et al., 2000), which has been shown to stabilize HIFs in cancer (Chan et al., 2002; Lee et al., 2011). Our lab showed that PAG-1 is a hypoxia-regulated gene in a series of cancer cell lines (Schörg et al., in preparation) and we could confirm induction at the mRNA level in Hep3B as well. Nevertheless, no significant changes in Epo mRNA levels were observed upon PAG-1 knockdown.

In conclusion, none of the analysed candidate factors showed specific effect on the newly discovered Epo 5'-HRE regulatory region under our experimental conditions. More extensive ChIP experiments would be required for some of the transcription factors, like USF-2 and the GATAs, to draw definitive conclusions on their effect on the 5'-HRE. ETV-4, ATF-4 and PAG-1 seem less promising candidate to further investigate. Since the *in vitro* model used is not appropriate to study renal Epo regulation, our lab is currently developing a transgenic mouse model expressing tamoxifen-inducible Cre recombinase under the control of a large 220 kb Epo locus (Epo-Cre) to be crossed with mice carrying floxed genes of interest. In this way, knockout of the candidate gene occurs specifically in Epo-producing cells, allowing *in vivo* investigation of the so far *in vitro* unavailable REPCs. Preliminary results obtained with Epo-Cre mice crossed with a Tomato reporter strain (*Rosa26-Stop-tdTomato*) displayed promising Tomato expression in peritubular, fibroblast-like cells in the kidney and co-localization with the interstitial fibroblast marker CD-73 , validating targeting of the renal Epo producing cells (unpublished data from Sara Santambrogio, Aurelia Lelli and Lisa Crowther) and recapitulating very recent results from the Yamamoto group with a non-inducible 180 kb Epo-Cre transgenic model (Yamazaki et al., 2013). This novel tool will allow in the future dissection of the role of the aforementioned candidate factors in Epo regulation in a relevant physiological context.

Figure 1

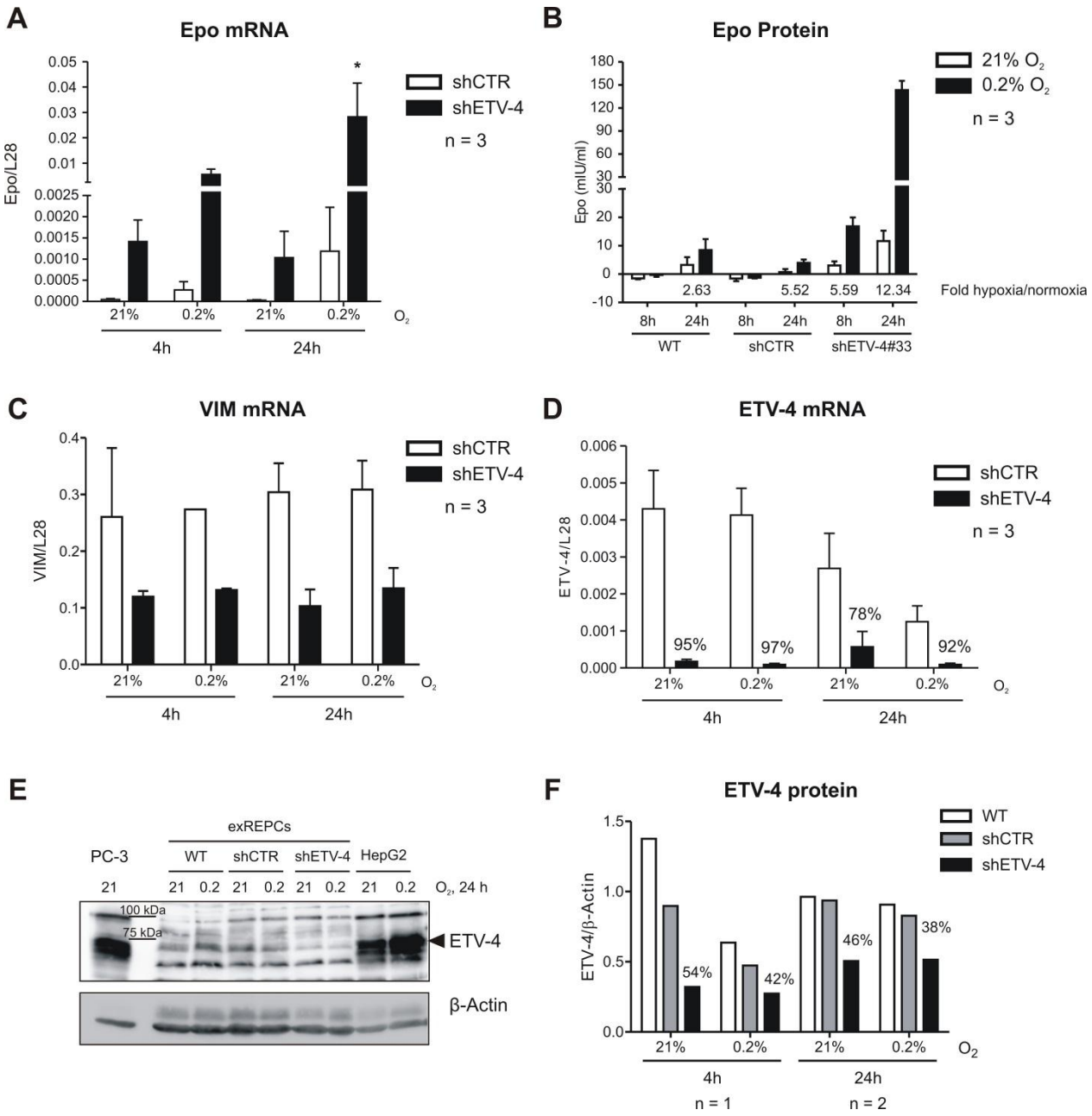

Figure 1 Stable ETV-4 knockdown in exREPCs was generated by means of lentiviral transduction. Wild type (WT), control (shCTR) and ETV-4 silenced (shETV-4) cells were exposed to 0.2% O_2 for the indicated time points and Epo mRNA (**A**) and protein (**B**) levels were measured by RT-qPCR and ELISA, respectively. Knockdown efficiency was evaluated by measuring VIM (**C**) and ETV-4 (**D**) mRNA, as well as ETV-4 protein levels (**E**. representative immunoblot, PC-3 cells are known to express high endogenous levels of ETV-4 and were used as a positive control; **F**. quantification of different experiments). Efficiency of the knockdown is

shown as % of shCTR cells. All data are expressed as mean ± SEM or mean alone. Statistics: 1-way ANOVA, Tukey post-test, *p < 0.05.

Figure 2

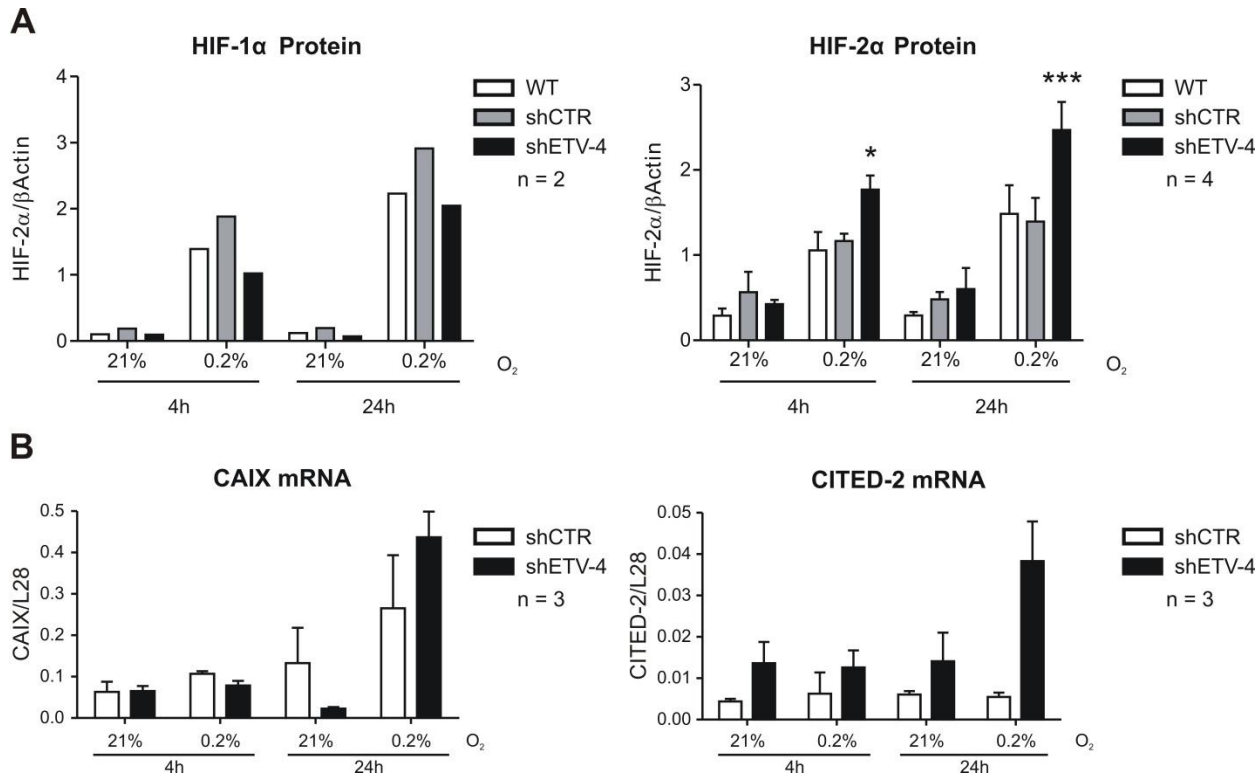

Figure 2 Wild type (WT), control (shCTR) and ETV-4 silenced (shETV-4) cells were exposed to 0.2% O_2 for the indicated time points and HIF-1α and -2α protein (**A**) levels were measured by immunoblotting (quantification of different experiments). HIF-target genes CAIX and CITED-2 mRNA levels were measured in shCTR and shETV-4 cells by RT-qPCR (**B**). All data are expressed as mean ± SEM or mean alone. Statistics: student's t-test, * = $p < 0.05$, ***$p < 0.001$.

Figure 3

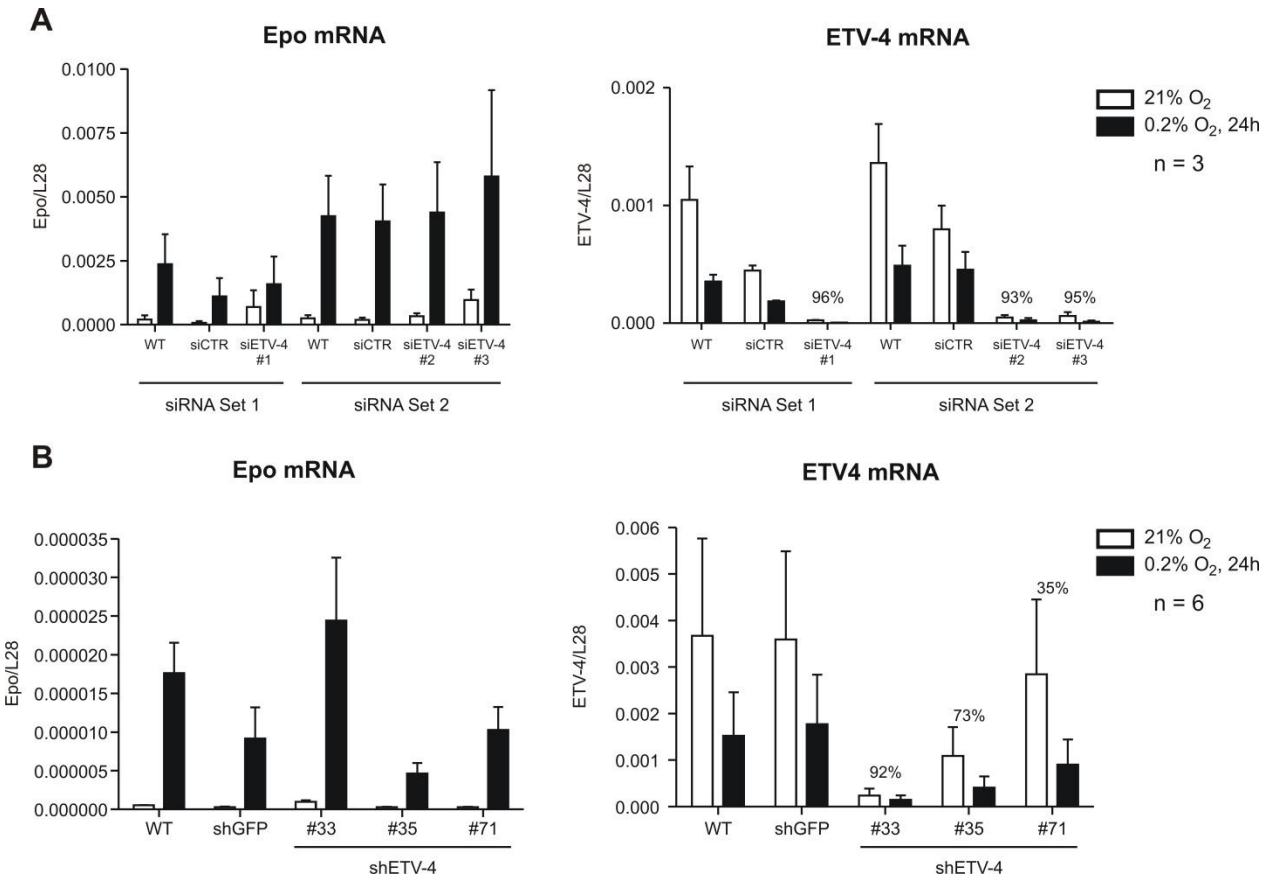

Figure 3 Transient (**A**) and stable (**B**) ETV-4 knockdown in exREPCs was generated by means of siRNA transfection and lentiviral transduction, respectively. Wild type (WT), control (siCTR/shGFP) and ETV-4 silenced (si/shETV-4) cells were exposed to 0.2% O_2 for 24 h and Epo and ETV-4 mRNA levels were measured by RT-qPCR. Efficiency of the knockdown is shown as % of siCTR/shGFP cells. All data are expressed as mean ± SEM.

Figure 4

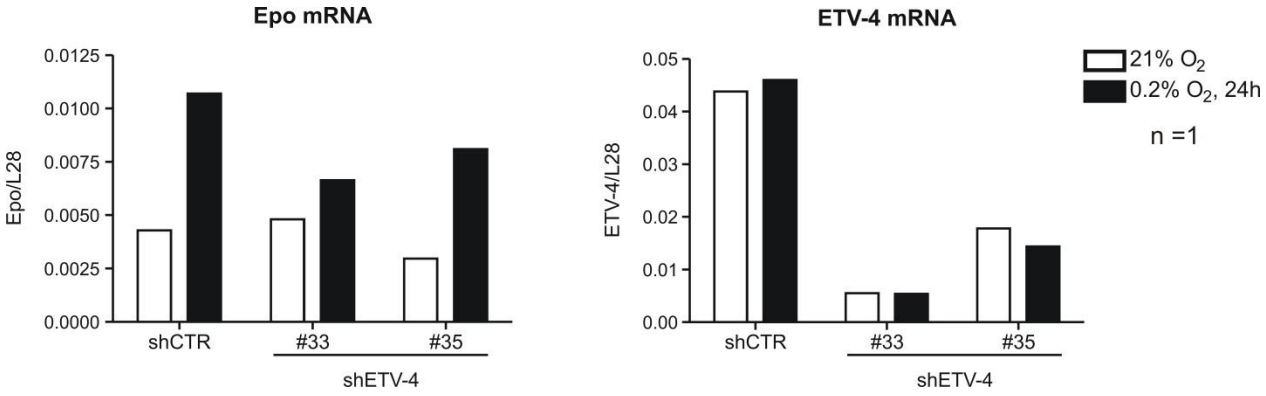

Figure 4 Stable ETV-4 knockdown in HepG2 was generated by means of lentiviral transduction. Control (shCTR) and ETV-4 silenced (shETV-4 #33 and #35) cells were exposed to 0.2% O_2 for 24 h and Epo and ETV-4 mRNA levels were measured by RT-qPCR.

Figure 5

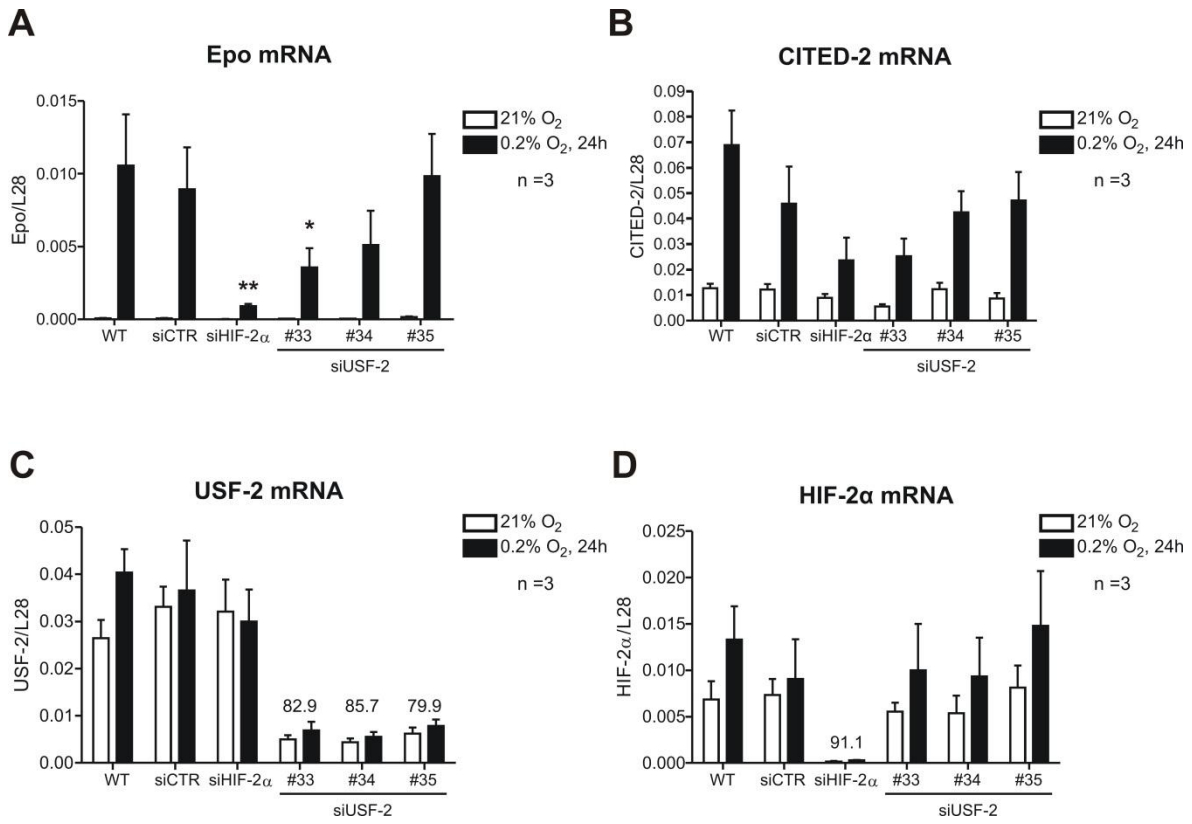

Figure 5 Transient USF-2 knockdown in Hep3B was generated by siRNA transfection. Wild type (WT), control (siCTR) and USF-2 silenced (siUSF-2) cells were exposed to 0.2% O_2 for 24 h and Epo (**A**), CITED-2 (**B**), USF-2 (**C**) and HIF-2α (**D**) mRNA levels were measured by RT-qPCR. Efficiency of the knockdown is shown as % of siCTR cells. All data are expressed as mean ± SEM. Statistics: Student's t-test, *$p < 0.05$, **$p < 0.01$.

Figure 6

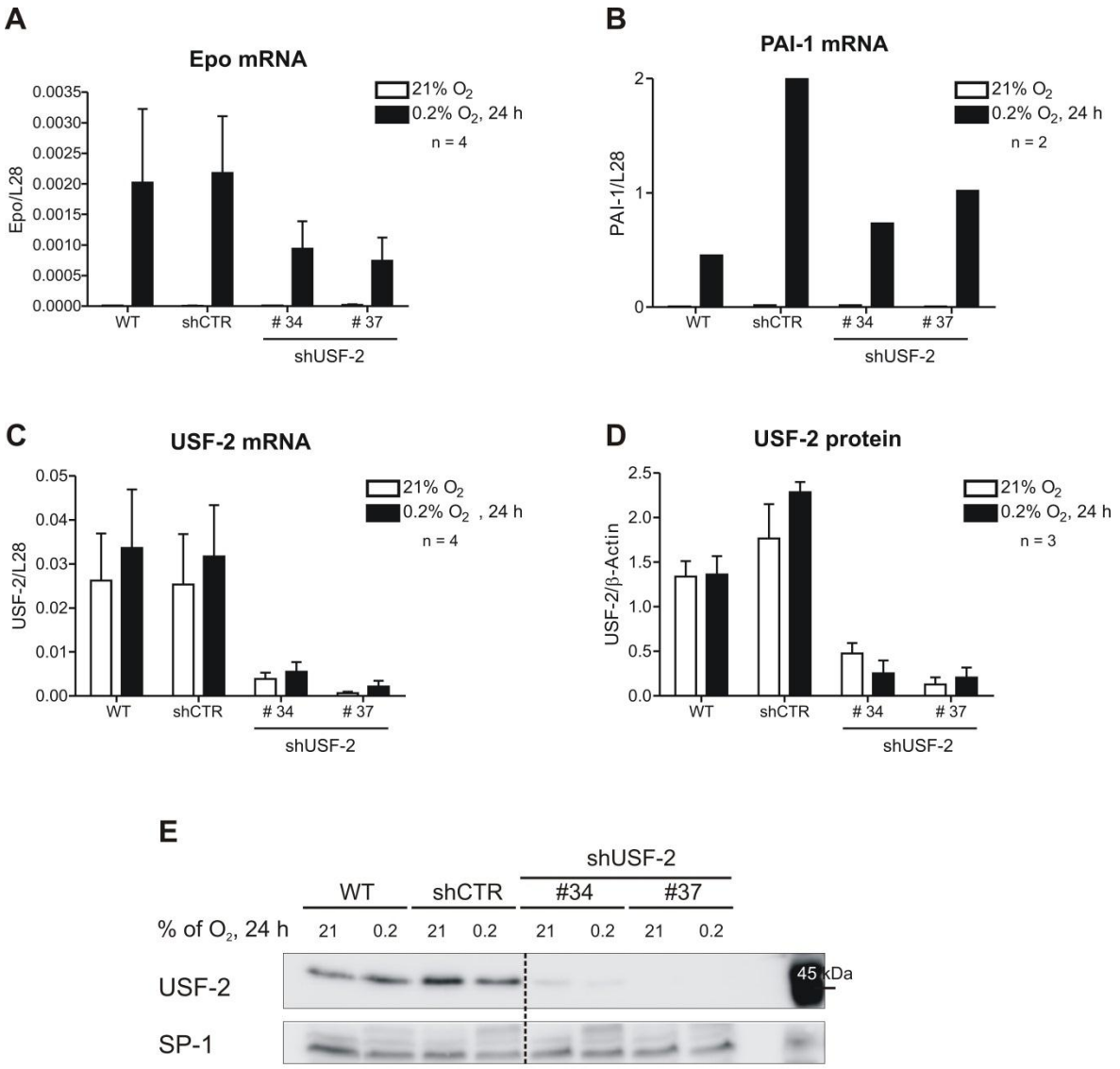

Figure 6 Stable USF-2 knockdown in Hep3B was generated by means of lentiviral transduction. Wild type (WT), control (shCTR) and USF-2 silenced (shUSF-2 #34 and #37) cells were exposed to 0.2% O_2 for 24 h and Epo mRNA (**A**), PAI-1 (**B**) and USF-2 (**C**) levels were measured by RT-qPCR. Knockdown efficiency was evaluated at the protein level as well (**E**. representative immunoblot, **D**. quantification of different experiments). All data are expressed as mean ± SEM or mean alone.

Figure 7

>hg19_dna range=chr7:100309066-100309368
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G**CACGTAT**GTGCTCCGGGCCTGAGCCCAGCAGAGGTGGTGGCAGGCAGCCTTTC
CCATCTGGTTTGTTTTGGCAGTTCCCTCCTGCAGCCTATTTGTTTGTTGTTTCT
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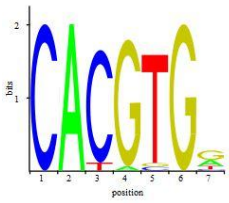

1 putative sites were predicted with these settings (80%) in sequence named hg19_dna

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0093.1	USF1	5.702	0.800786337596817	110	116	1	CACGTAT

Figure 7 Sequence of human genomic DNA surrounding the novel Epo 5'-HRE on the long arm of chromosome 7 (UCSC genome browser): the HRE is underlined (CACA repeat + HBS) and the putative USF binding site is highlighted in blue. Prediction of USF binding site was performed using the JASPAR database with a relative profile score threshold of 80 % (http://jaspar.genereg.net/cgibin/jaspar_db. pl?rm=browse&db= core&tax_group=vertebrates).

Figure 8

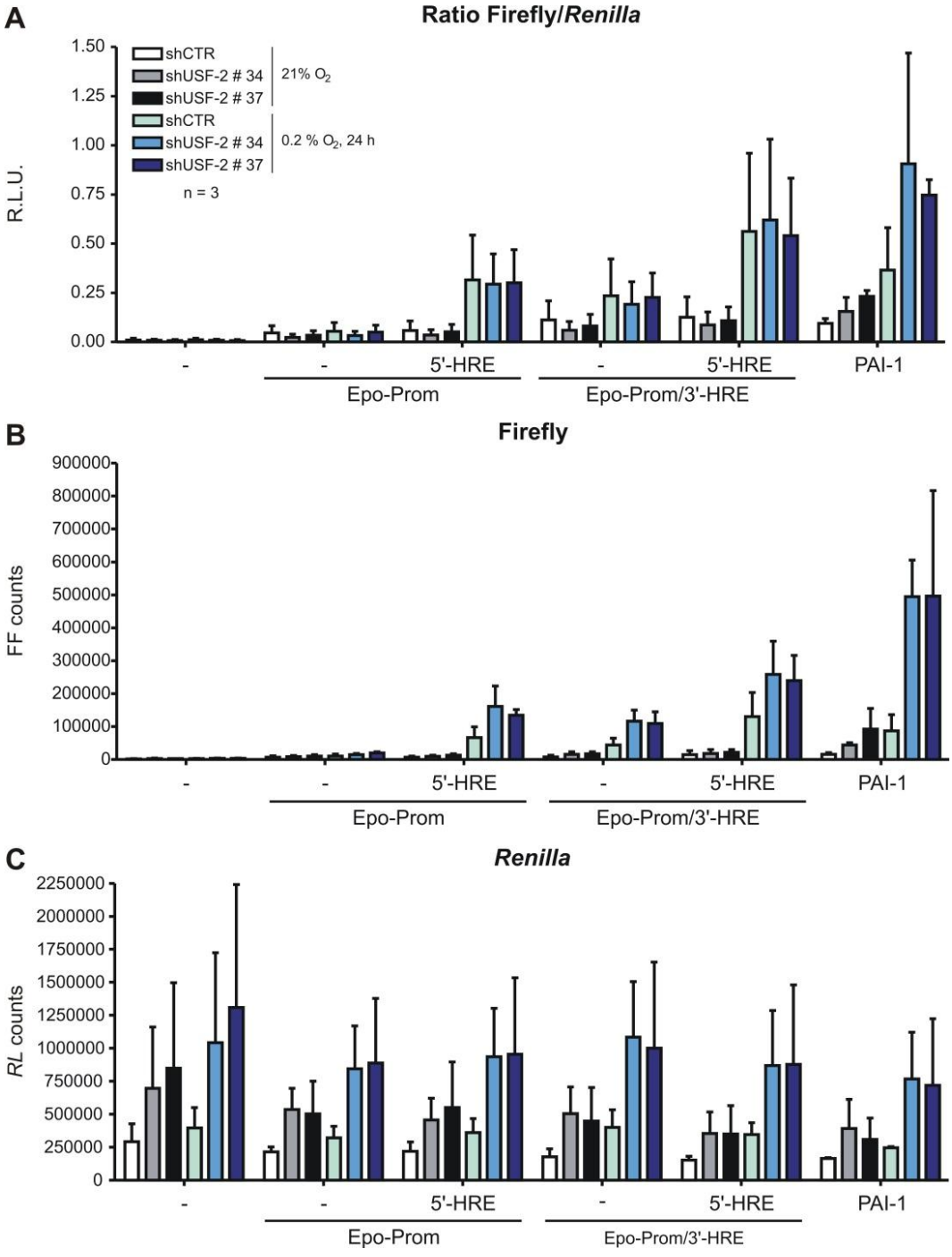

Figure 8 Control (shCTR) and USF-2 silenced (shUSF-2 #34 and #37) Hep3B were co-transfected with the indicated firefly constructs (see chapter 3) and an SV40-driven *Renilla* construct as transfection control. Results are displayed as R.L.U. (**A**), firefly counts (**B**) or *Renilla* counts (**C**); all data are expressed as mean ± SEM.

Figure 9

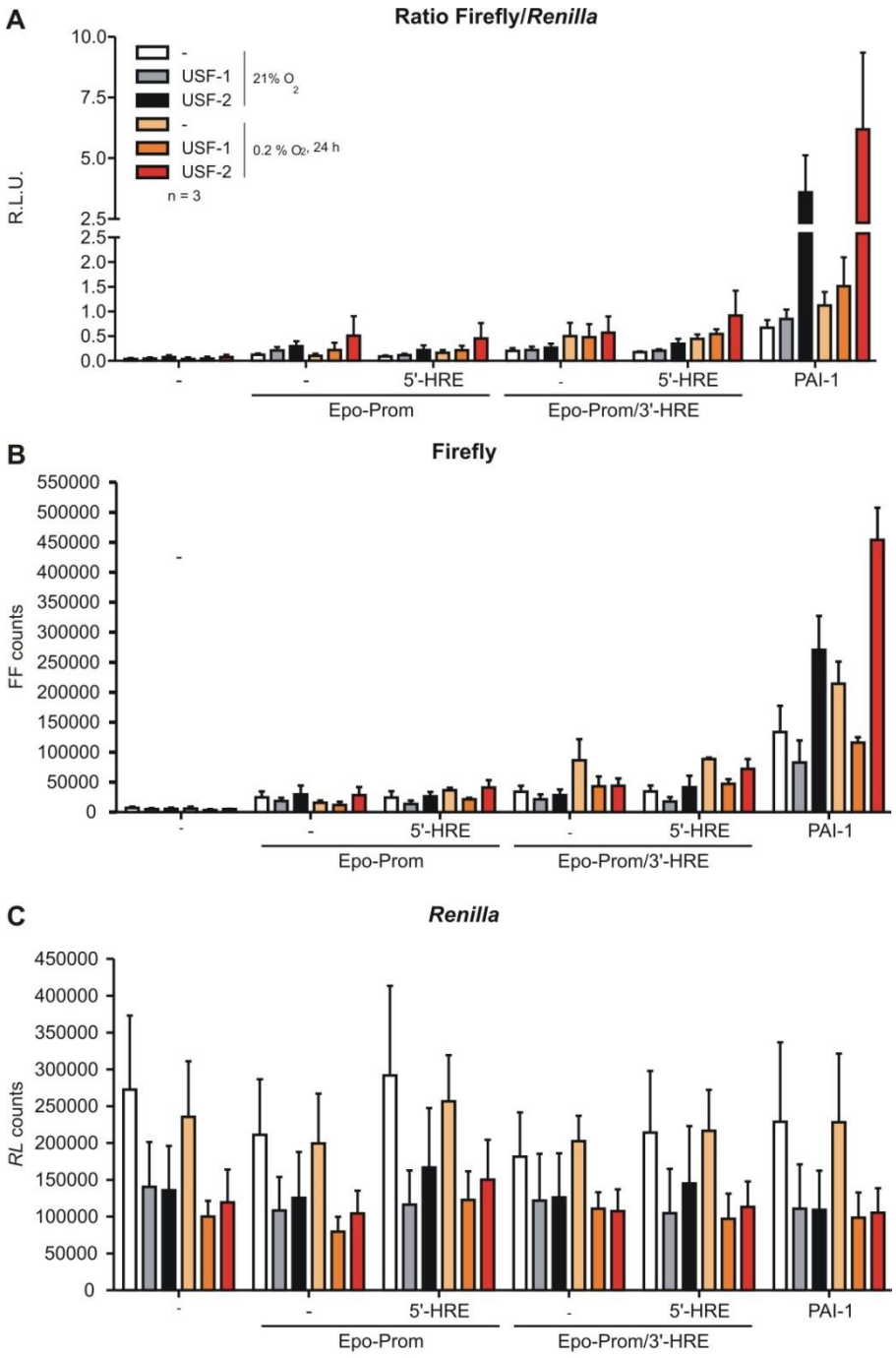

Figure 9 The indicated firefly constructs (see chapter 3) were co-transfected in Hep3B with an empty control or a USF-1/-2 overexpressing vector and an SV40-driven *Renilla* construct as transfection control. Results are displayed as R.L.U. (**A**), firefly counts (**B**) or *Renilla* counts (**C**); all data are expressed as mean ± SEM.

Figure 10

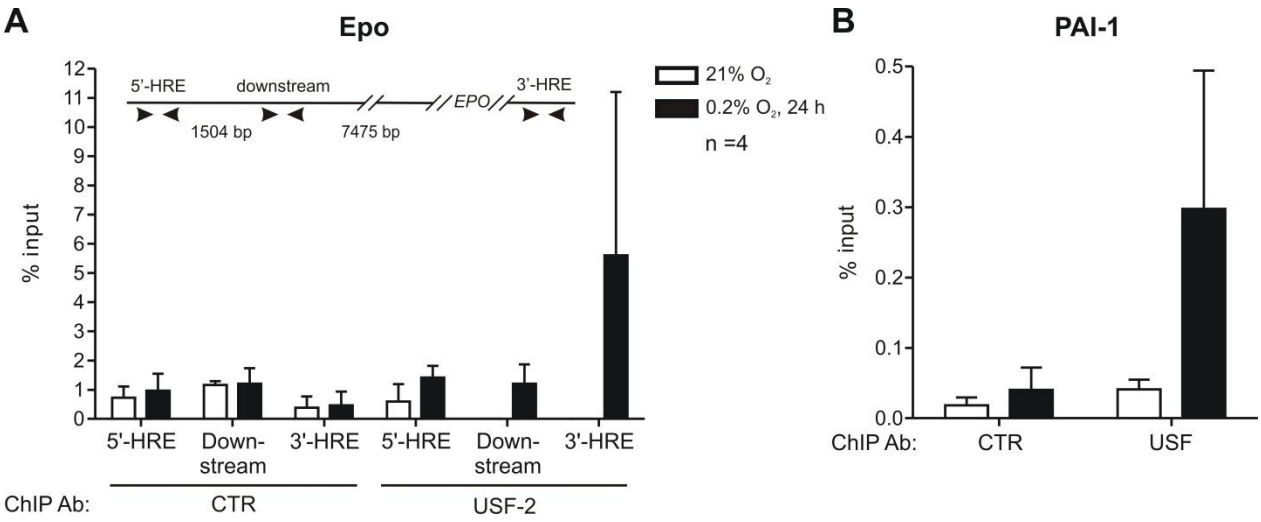

Figure 10 ChIP was performed in normoxic or hypoxic (0.2% O_2, 24 h) Hep3B cells using control (CTR) or USF-2 specific antibodies. (**A**) The amount of co-precipitated chromatin derived from human Epo 5'-HRE, its downstream region and 3'-HRE was determined by qPCR. The scheme depicts the different primer regions used for the experiment. (**B**) PAI-1 promoter was used as additional positive control. Data are expressed as mean ± SEM of chromatin input %.

Figure 11

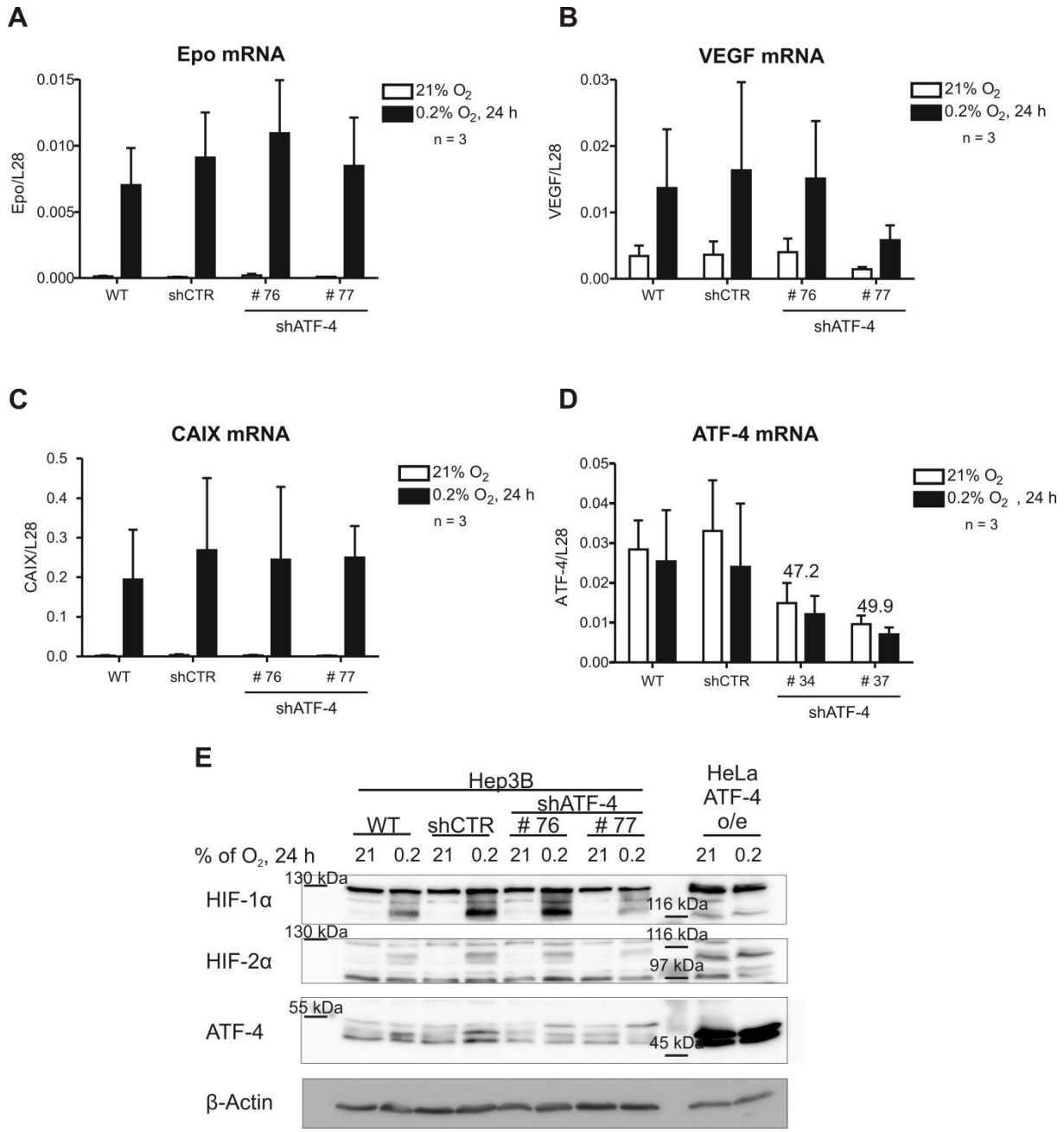

Figure 11 Stable ATF-4 knockdown in Hep3B was generated by means of lentiviral transduction. Wild type (WT), control (shCTR) and ATF-4 silenced (shATF-4 #76 and #77) cells were exposed to 0.2% O_2 for 24 h and Epo mRNA (**A**), VEGF (**B**), CAIX (**C**) and ATF-4 (**D**) levels were measured by RT-qPCR. Knockdown efficiency was evaluated at the protein level as well using HeLa cells transfected with an ATF-4 overexpressing plasmid (o/e) as positive control (**E.** representative immunoblot). All data are expressed as mean ± SEM.

Figure 12

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TCCGGGCCTGAGCCCAGCAGA**GGTGGTGGC**AGGCAGCCTTTCCCATCTGGTTTGTTTTGG
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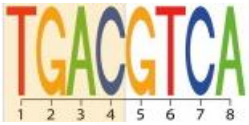
AAG

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>Mouse contig1:1-314 (-)

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000000061  TAGCCGAGCGGGGAGCCGGCAGGAAGCT-GGATGGAGCTGTGTCTCCCTGCACGTATGTG  000000119
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000000120  --------------CTCCGGGCCTGAGCCCAGCAGAGGTGGTGGCAGGCAGCCTTT----  000000161
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000000162  ------------CCCATCTGGTTTGTTTTGGCAGTTCCCT----CCTGCAGCCTATTTGT  000000205
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000000206  TTGTTGTTTCTACTCCATGTTTTCTCACTGCTTGCCAGTGGGGCTTCCCTCAGTGGCAGT  000000265
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Figure 12 Sequence of human genomic DNA surrounding the novel Epo 5'-HRE on the long arm of chromosome 7 (UCSC genome browser): the HRE is underlined (CACA repeat + HBS) and the putative ATF-4 binding site is highlighted in green. Prediction of ATF-4 binding sites was performed using the rVista webserver (http://genome.lbl.gov/vista/rvista/submit.shtml) based on homology between human and mouse genomic sequences.

Figure 13

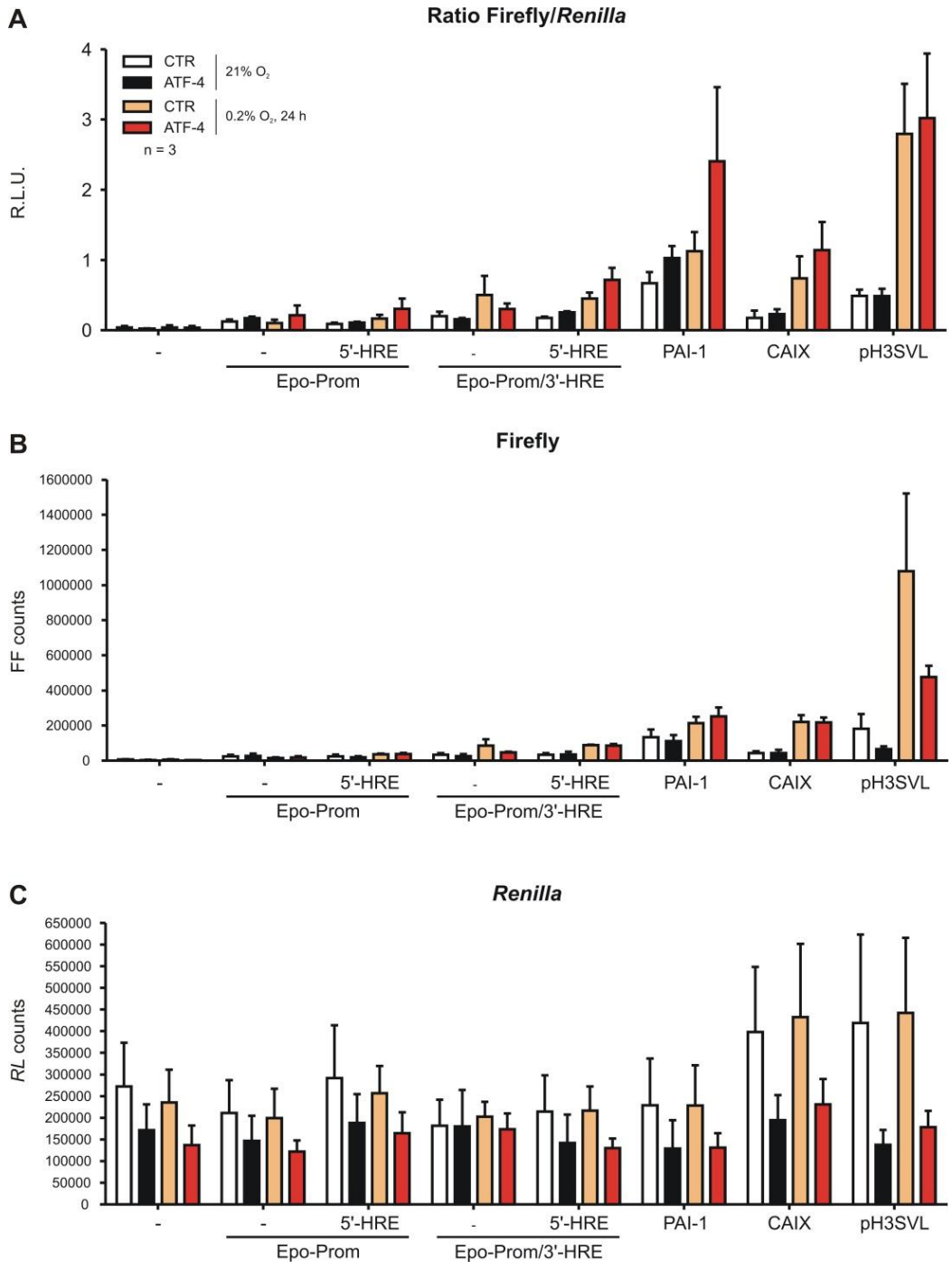

Figure 13 The indicated firefly constructs (see chapter 3) were co-transfected in Hep3B with an empty control or an ATF-4 overexpressing vector and an SV40-driven *Renilla* construct as transfection control. Results are displayed as R.L.U. (**A**), firefly counts (**B**) or *Renilla* counts (**C**); all data are expressed as mean ± SEM.

Figure 14

>hg19_dna range=chr7:100309066-100309368
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GGTTTGTTTTGGCAGTTCCCTCCTGCAGCCTATTTGTTTGTTGTTTCTAC**TCCATG**
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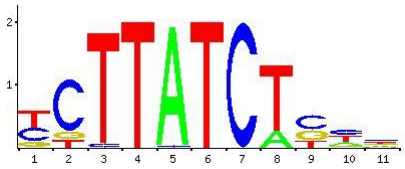

3 putative sites were predicted with these settings (80%) in sequence named hg19_dna

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
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MA0482.1	Gata4	4.676	0.839018043625829	223	233	1	TGTTTTCTCAC
MA0482.1	Gata4	4.799	0.840786452867327	225	235	1	TTTTCTCACTG

Figure 14 Sequence of human genomic DNA surrounding the novel Epo 5'-HRE on the long arm of chromosome 7 (UCSC genome browser): the HRE is underlined (CACA repeat + HBS) and the putative GATA factors binding site is highlighted in red. Prediction of GATA factors binding site was performed using the JASPAR database with a relative profile score threshold of 80 % (http://jaspar.genereg.net/cgibin/jaspar_db.pl?rm=browse&db=core&tax_group=vertebrates).

Figure 15

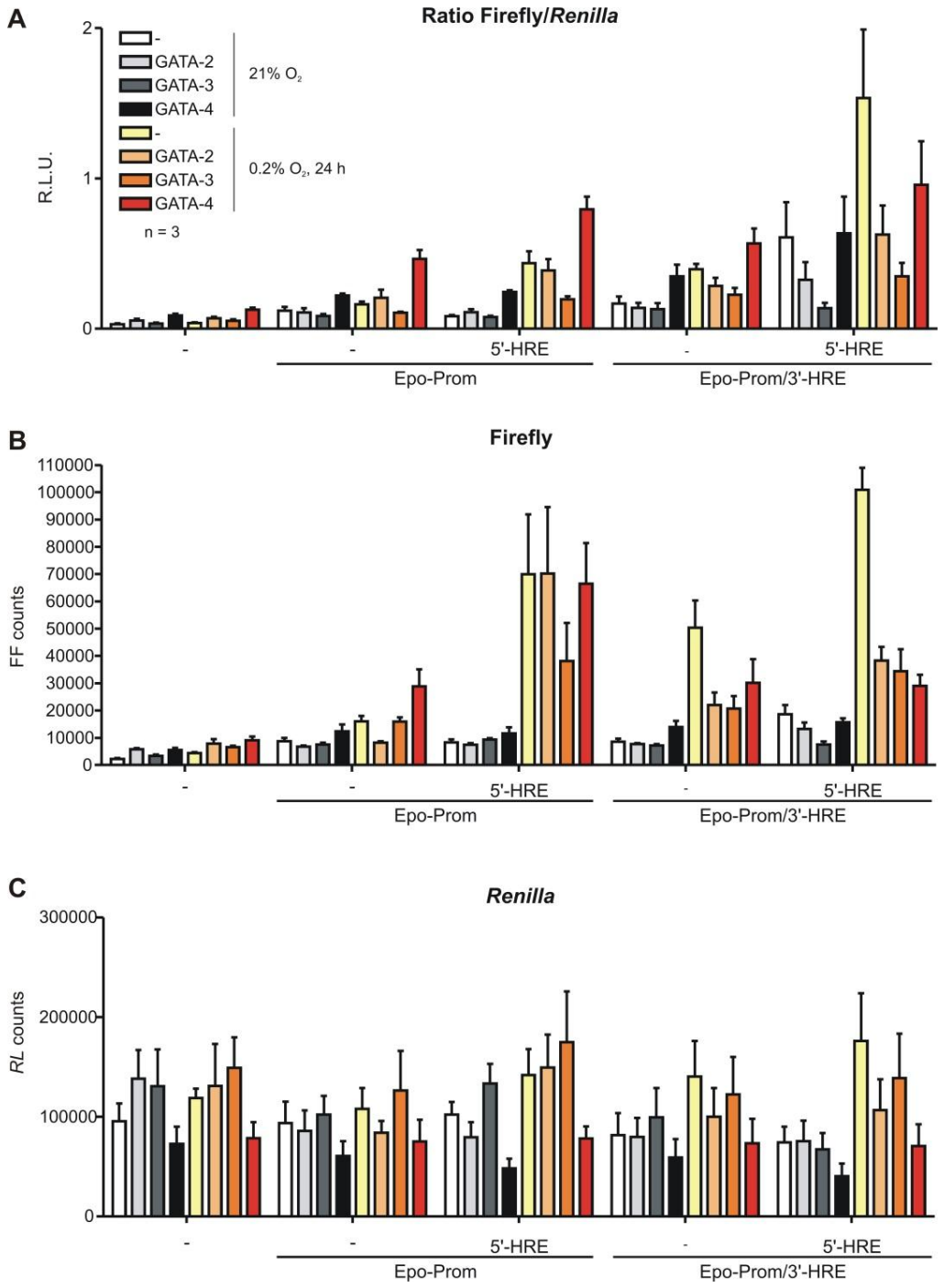

Figure 15 The indicated firefly constructs (see chapter 3) were co-transfected in Hep3B with an empty control or a GATA-2, -3 or -4 overexpressing vector and an SV40-driven *Renilla* construct as transfection control. Results are displayed as R.L.U. (**A**), firefly counts (**B**) or *Renilla* counts (**C**); all data are expressed as mean ± SEM.

Figure 16

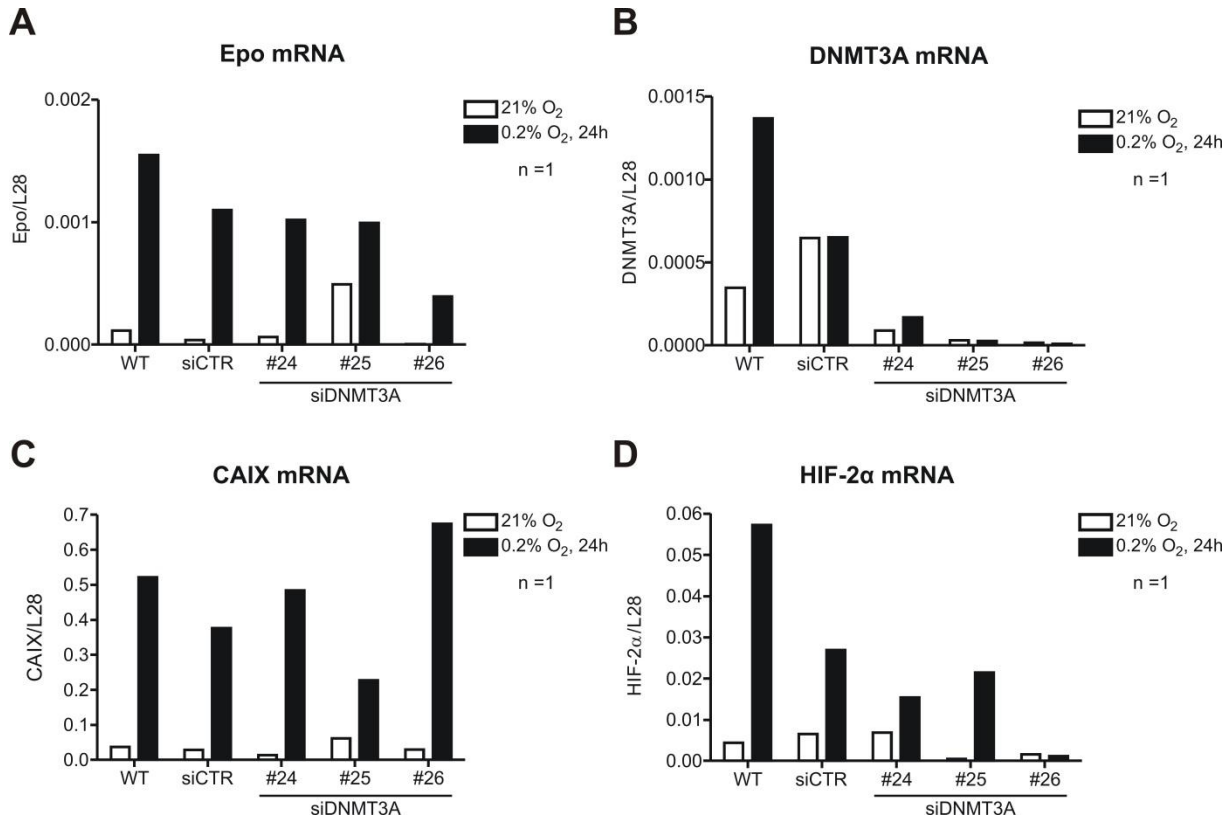

Figure 16 Transient DNMT3A knockdown in Hep3B was generated by siRNA transfection. Wild type (WT), control (siCTR) and DNMT3A silenced (siDNMT3A) cells were exposed to 0.2% O_2 for 24 h and Epo (**A**), DNMT3A (**B**), CAIX (**C**) and HIF-2α (**D**) mRNA levels were measured by RT-qPCR. All data are expressed as mean alone.

Figure 17

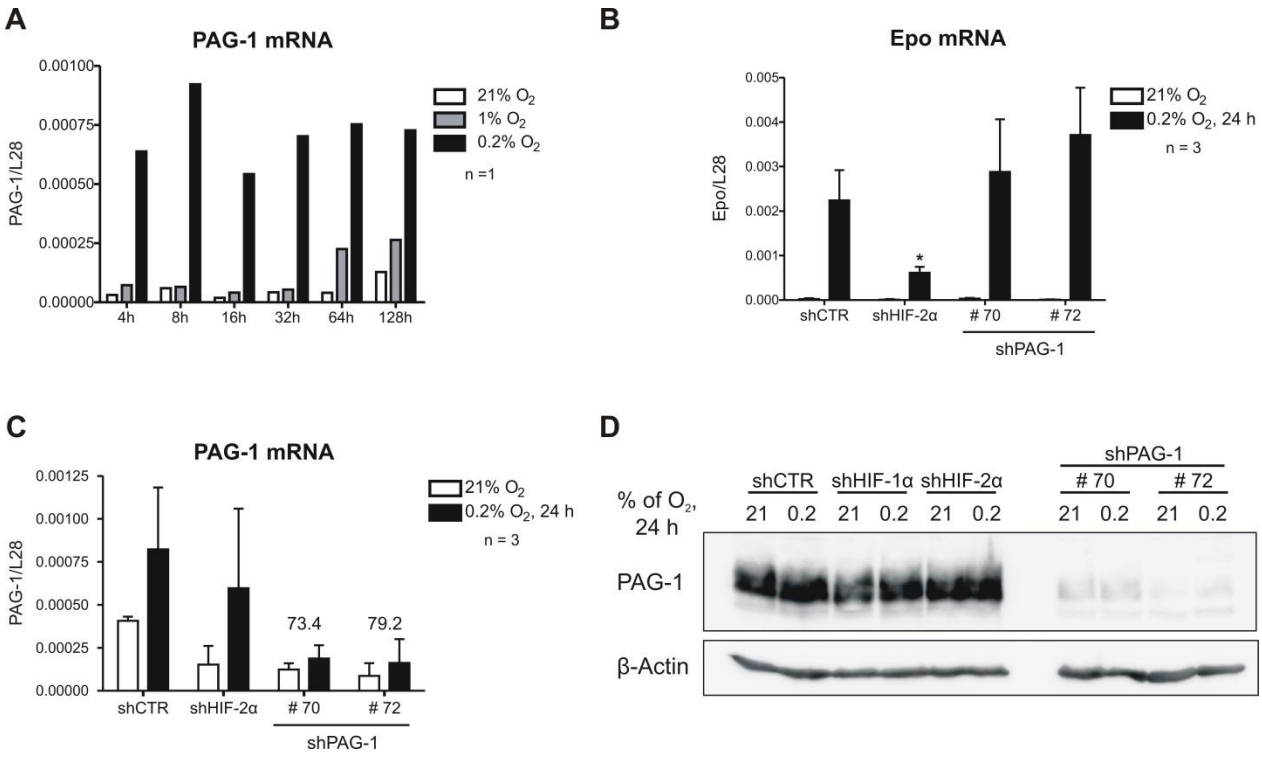

Figure 17 PAG-1 expression was measured at the mRNA level by RT-qPCR in exREPCs exposed to 21, 1 or 0.2% O_2 for the indicated time points (**A**). Stable PAG-1 knockdown in exREPCs was generated by means of lentiviral transduction. Control (shCTR) and PAG-1 silenced (shPAG-1 #70 and #72) cells were exposed to 0.2% O_2 for 24 h and Epo mRNA (**B**) and PAG-1 (**C**) levels were measured by RT-qPCR. Efficiency of the knockdown is shown as % of shCTR and was evaluated at the protein level as well (**D.** representative immunoblot). All data are expressed as mean ± SEM or mean alone.

References

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7. Epo regulatory peptides

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Introduction

Accumulating evidence suggests the possibility for erythropoietin (Epo) to be regulated by peptides and hormones in the blood stream (see introduction, paragraph 1.5). The aim of this sub-project was to test *in vitro* putative Epo regulatory peptides, selected on the basis of available literature data and corresponding receptor expression in Hep3B. Tested candidates included extracts from hypophysis (bovine pituitary extract, BPE) and the steroid hormones aldosterone (ALDO) and β-estradiol (E2). Our approach was additionally complemented by the screening of a human haemofiltrate (HF)-derived peptide collection (Schulz-Knappe et al., 1997) using hypoxia-inducible factor (HIF)-reporter cell lines in order to identify novel regulators of the HIF pathway and, possibly, of Epo production too. Details on the rationale of candidate selection and on the chosen strategy are given in the result section.

Materials and methods

Cell culture, reagents and hormone treatment

ExREPCs, Hep3B, HepG2, HK-2 and Hek293T were cultured in DMEM (high glucose, Sigma-Aldrich) supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Hypoxia experiments were carried out at the indicated concentration of oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies). Dimethyloxaloylglycine (DMOG, Frontier Scientific) was dissolved in dimethyl sulfoxide (DMSO) and used at the concentration of 1 mM for 8 h. Deferoxamine (DFX, Sigma-Aldrich) and cobalt chloride ($CoCl_2$, Sigma-Aldrich) were dissolved in water and used at a concentration of 100 µM. For treatment with selected peptides, Hep3B cells were plated at a density of 4×10^5 cells per single well of a 6-well plate. The day after, medium was changed to

non-FCS containing medium for 16 h, followed by hormone stimulation and exposure to 21% or 0.2% of O_2 for 24 h (ALDO and E2) or 48 h (BPE). BPE was purchased by Lonza (CC-4009, stock concentration: 13 mg/ml) and diluted in FCS-free medium at the indicated concentrations; bovine serum albumin (BSA, Sigma-Aldrich) was re-suspended in PBS at 13 mg/ml and diluted in FCS-free medium at the indicated concentrations. ALDO (Sigma-Aldrich) was re-suspended in ethanol at 100 µM and diluted in FCS-free medium at the indicated concentrations. E2 (Sigma-Aldrich) was re-suspended in ethanol at 73.4 µM and diluted in FCS-free medium at the indicated concentrations. ICI 182,780 (or Fulvestrant, (7α, 17β- [9- [(4, 4, 5, 5, 5 – Pentafluoropentyl) sulfinyl] nonyl] estra-1, 3, 5 (10) -triene-3, 17-diol, Sigma-Aldrich) was re-suspended in ethanol at 33 µM and diluted in FCS-free medium at the indicated concentration.

RNA extraction and quantitative PCR

RNA was extracted by using the phenol-chloroform method and cDNA was generated by reverse transcription (RT) of 2 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were quantified by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) in combination with a MX3000P light cycler (Agilent) or by conventional RT-PCR (for hV1a and hV2) and the primers listed in table 1. Initial template concentrations were calculated by comparison with serial dilutions of a calibrated standard. Ribosomal protein L28 mRNA levels were used to normalize the data. cDNA from human kidney samples was kindly provided by Prof. Olivier Devuyst (University of Zürich, Switzerland).

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size
hV1a	CGGCTTCATCTGCTACAACATC	CGAGTCCTTCCACATACCCGT	505 bp
hV2	TGGTCGTCTATGTGCTGTGC	GCATAGATCCAGGGGTTGGT	148 bp
hL28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198 bp
hEpo	TCACTGTCCCAGACACCAAA	CCTCCCCTGTGTACAGCTTC	362 bp
hPAI-1	ACTGGAAAGGCAACATGACC	GAGGAAGGGTCTGTCCATGA	296 bp
hCAIX	GGGTGTCATCTGGACTGTGTT	CTTCTGTGCTGCCTTCTCATC	309 bp
hPGK-1	AACAAGGTTAAAGCCGAGCC	TGAGCTGGATCTTGTCTGCA	259 bp
hGLUT-1	TCACTGTGCTCCTGGTTCTG	CCTGTGCTCCTGAGAGATCC	233 bp
hMR	CCAACTTCAGGCTGCTCAGA	GCTCCACAGCCTGAGAAACT	290 bp
hGR	GCAGTGGAAGGTAGACAGCA	CTCCAACAGTGACACCAGGG	273 bp
hERβ	GTCAGGCATGCGAGTAACAA	ATTTTCGTTTCTCCCGAGGG	230 bp
hPCK	AGGCGGCTGAAGAAGTATGA	ACGTAGGGTGAATCCGTCAG	299 bp
hERα	AGACATGAGAGCTGCCAACC	GCCAGGCACATTCTAGAAGG	299 bp

Table 1 Primers used for qPCR amplification

Immunoblotting

Combined cytoplasmic and nuclear extracts were prepared using a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 x protease inhibitory cocktail (Sigma). Protein concentrations were determined by the bicinchoninic acid assay method and up to 80 µg of cellular protein was subjected to immunoblot analyses. Membranes were probed with antibodies against ERα (sc-542, Santa Cruz Biotechnology) and β-actin (Sigma). Signals from HRP-coupled secondary antibodies were detected with ECL substrate (all Pierce) using a luminescent image analyser (LAS-4000, FUJIFILM). β-actin was used as loading control.

ERα overexpression

Hep3B cells were plated at a density of 1.5×10^6 cells per 10 cm dish (for RNA extraction) and 3.5×10^6 cells per 15 cm dish (for protein extraction) and transiently transfected after 24 h with 3 µg of pCMV5-hERα (kindly provided by Prof. Alex Odermatt, University of Basel, Switzerland) or pEGFP-C1 (Clontech) as a control, using polyethylenimine (PEI). 24 h post transfection cultures were evenly split onto 6-well plates or 10 cm dishes, respectively, and after 8 h medium was changed to FCS-free medium for 16 h. Cells were then exposed to the indicated concentration of E2 and oxygen for an additional 24 h.

Reporter cell lines

For the first screening round (figure 9), exREPCs were plated at a density of 2.5×10^4 cells per single well of a 96-well plate and transiently transfected after 24 h with 5 ng pH3SVL and 0.5 ng pRL-SV40 using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). For the second screening round (figure 11), stable reporter Hep3B were generated by electroporation of 50 µg of linearized pH3SVL, pSV2Neo (containing a neomycin resistant cassette) and pRL-SV40-hygro (containing a hygromycin resistant cassette) and cultured in DMEM supplemented with 10% heat-inactivated FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 1 mg/ml G418 (PAA) and 10 µg/ml hygromycin B (Calbiochem). Other reporter cell lines (PHD2, Epo 5'-HRE and Epo reporter) were generated by lentiviral (LV) transduction. LV expression vectors pDestX1-puro and pDestX1-neo were kindly provided by Prof. Ian Frew (University of Zürich, Switzerland) and used for recombination with entry vectors containing firefly and *Renilla* genes under the control of the regulatory regions depicted in figure 10A and 12 (all from Invitrogen). Viral particles were produced in Hek293T cells by co-transfection of the respective transfer vector (3 µg) with the packaging plasmids pLP1 (4.2 µg), pLP2 (2 µg) and

pVSV-G (2.8 µg, all from Invitrogen) using PEI transfection as described before (Stiehl et al., 2012). Hep3B cells were transduced with lentiviral-pseudotyped particles and cell pools were derived by puromycin selection.

Peptide collection screening and luciferase assay

A lyophilized peptide collection of 384 fractions, each corresponding to 5 L HF-equivalent, was prepared as previously described (Schulz-Knappe et al., 1997). For the first screening round (figure 9), each fraction was re-suspended in 200 µl of sterile water and stored at -20°C. 2 µl of thawed fractions were added every 2 h to 96-well plates containing reporter Hep3B cells and 100 µl of FCS-free medium per well, for 8 h (4 total additions) in normoxia and hypoxia. For the second screening round (figure 11), each fraction was re-suspended in 40 µl of sterile water (STOCK concentration), diluted 1:2 and 1:4 with sterile water and used immediately for experiment with the same protocol used for the first screening round. Water alone was used as internal control for each experiment. After 8 or 24 h of hypoxia (see protocol scheme, figure 9 and 10), cells were lysed with 20 µl of passive lysis buffer (PLB) and luciferase activities of triplicate wells were determined using the Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Reporter activities were expressed as relative firefly/*Renilla* luciferase activities.

Results

BPE

In vivo experiments suggest a direct link between the pituitary gland, or hypophysis, and renal Epo production. Hypoxia in the brain stem of rats results in increased Epo plasma level in a hypophysis- and kidney-dependent manner (von Wussow et al., 2005) and the pituitary hormone ADH (antidiuretic hormone or arginine vasopressin, AVP) was able to induce Epo when administrated to normoxic rats (Engel and Pagel, 1995). The hypophysis is located at the base of the brain, just below the hypothalamus, and is divided into an anterior and a posterior part. The anterior pituitary gland secretes a number of hormones under the control of hypothalamic releasing hormones, while the posterior part belongs to the brain itself since it contains the nerve endings of the supraoptic and paraventricular neurons of the hypothalamus. ADH is produced by the posterior hypophysis as a consequence of increased blood osmolality or decreased blood volume (BV) and is essential for cardiovascular and renal homeostasis. ADH promotes in fact

vasoconstriction, in order to increase blood pressure, and water re-absorption by the distal nephron, thereby resulting in increased BV (Boron and Boulpaep, 2005). 5 types of ADH receptors have been identified so far, all belonging to the family of G-protein coupled receptors (GPCRs); the most well-known are the vascular V1 and the renal V2 receptors (Holmes et al., 2003). Upon ligand binding, V2 receptors mediate fusion of aquaporin-2 (AQP-2)-containing vesicles with the apical membrane of principal cells in the collecting duct and increase water retention (Boron and Boulpaep, 2005). According to the aforementioned report (Engel and Pagel, 1995), ADH administration to rats increased Epo plasma levels via V1a receptor subtype without influencing glomerular filtration rate or renal blood flow, suggesting that renal vasoconstriction and subsequent hypoxia are not the cause of the observed rise in Epo levels. Therefore, we hypothesized a direct effect of ADH on Epo-producing cells.

In order to test our hypothesis, we evaluated the expression of V1a and V2 receptors in our *in vitro* model, Hep3B cells, alongside with a panel of cell lines and cDNA extracted from human kidney and the proximal tubule cell line HK-2 as a positive control. RT-PCR revealed no expression of V1a in any of the cell lines tested and low expression of the V2 longer transcript variant (figure 1). Given that ADH can bind to both receptors and that the hypophysis-dependent effect on Epo could be mediated by other pituitary hormones, we treated Hep3B cells with a homogenized extract from bovine pituitary gland (BPE), which most likely contains a representation of hypophysis-derived peptides, and measured mRNA levels of HIF-2 and HIF-1 target genes. HIF-2 specific Epo and PAI-1 (plasminogen activator inhibitor type 1) mRNA levels were unchanged between BPE-treated and control cells, as shown in figure 2; HIF-1 target genes, such as CAIX (carbonic anhydrase 9), PGK-1 (phosphoglycerate kinase 1) and GLUT-1 (glucose transporter 1), did not show changes in transcript levels either (figure 3). CAIX mRNA levels tended to increase with high doses of BPE, both in normal and reduced oxygen conditions, but the effect was not statistically significant (figure 3A). We concluded that Hep3B express the V2 receptor, at least on mRNA level, but treatment with BPE does not lead to transcriptional changes in the HIF target genes measured under these experimental conditions.

Steroid hormones

Steroid hormones (SHs) are produced by the adrenal gland and by the gonads and share the same precursor, cholesterol, and the same basic ring structure. SHs can be divided into 5 major classes: progestogens, glucocorticoids, mineralocorticoids, androgens and estrogens, exerting a wide variety of functions within the human body. Due to their lipophilic nature, SHs can diffuse into the cell membrane and bind to their intracellular receptors, which are transcription factors.

Upon binding, steroid hormone receptors (SHRs) form homodimers and activate transcription of target genes containing the corresponding SH-responsive element in the regulatory regions (Alberts et al., 2002). As already discussed in the introduction, most of the data on SH-dependent Epo regulation involve sexual hormones (Alexanian, 1969; Mukundan et al., 2002; Mukundan et al., 2004), but no direct link between SHs and *EPO* transcriptional regulation has been characterized so far.

We started to study the expression profile of different SHRs in Hep3B cells. Mineralcorticoid (MR), glucocorticoid (GR) and estrogen receptor β (ERβ) mRNA was expressed by the Epo-producing cell model, albeit at low levels (figure 4). We could not detect estrogen receptor α (ERα), progesterone receptor (PgR) and androgen receptor (AR) transcripts (data not shown).

Based on these results, we treated Hep3B cells with different doses of the MR high affinity agonist ALDO. ALDO is physiologically produced by the adrenal gland and can bind to GR as well with lower affinity. It plays a central role in the renin-angiotensin system, its release being increased by angiotensin II, and it acts on principal cells of the collecting duct. ALDO promotes sodium and water reabsorption and potassium secretion, thereby increasing BV and blood pressure. Since ALDO is a crucial regulator of BV and Epo controls the main cellular component of the blood, erythrocytes, we hypothesized a potential role for ALDO in Epo regulation. Figure 5 displays the results obtained with ALDO treatment of Hep3B: no significant changes could be observed in Epo mRNA levels, even though a trend of increased Epo production could be measured with the highest (but not physiological) doses of the hormone. CAIX and PCK (phosphoenolpyruvate carboxykinase) were used as controls for hypoxic and ALDO exposure, respectively. Of note, PCK mRNA was significantly increased only with the highest dose of ALDO.

More data are available concerning the inhibitory effect of estrogens on Epo synthesis that could explain the differences in haematocrit (Hct) levels between men and women (see introduction). Experiments with Epo reporters in Hep3B showed decreased reporter activity and Epo protein levels upon treatment with the ER agonist E2 (Mukundan et al., 2004). Moreover, E2 treatment of ER-positive breast cancer cell lines, like MCF-7, resulted in decreased HIF-2α mRNA and protein levels, both in normoxic and hypoxic conditions, further suggesting a link between estrogen activity and Epo regulation (Jerry H. Fuady, unpublished data). Since Hep3B expressed ERβ in our hands, we treated these cells with different doses of E2 and the ER antagonist ICI and investigated a potential effect on endogenous Epo mRNA. When considering Epo hypoxia/normoxia fold induction, a trend of increased Epo folds could be observed with the

lowest E2 concentration and was not present when cells were treated with ICI (figure 6A). However, the vehicle control (ethanol alone) gave the strongest reduction in Epo mRNA, affecting the interpretation of the whole experiment. The hypoxic control CAIX transcript levels resulted to be more stable compared to Epo mRNA levels regardless of E2 or ICI treatment (figure 6B). We were not able to recapitulate the MCF-7-based results: HIF-2α mRNA levels were not decreased upon treatment of Hep3B with E2 (figure 6C). PgR is known to be a transcriptional target of ERs in breast cancer cell lines (Horwitz et al., 1978; Otto, 1995), but no PgR mRNA could be detected after estrogen treatment in Hep3B (data not shown): we thus lacked a suitable positive control for ER activation. Given the fact that ERα is not expressed in Hep3B but could mediate the effect of E2 on Epo as well, we decided to transiently overexpress this ER isoform prior to E2 treatment in our cell model. Again, no significant effect on Epo or HIF-2α mRNA could be observed after ERα overexpression and treatment with E2 (figure 7A and B). Transfection efficiency was relatively low, as demonstrated by GFP expression in the control cells (figure 7D); nevertheless ERα could be measured on mRNA and protein level specifically in transfected Hep3B cells (figure 7C and D). Collectively, these data suggest no clear effect of E2 on *EPO* or *HIF2A* transcription in Hep3B cells, at least under our experimental conditions.

Screening of a human HF-derived peptide collection

Since no Epo regulatory peptide has been identified up to now, we decided to screen a complete human HF-derived peptide collection (Schulz-Knappe et al., 1997) for the effect on the HIF pathway and, possibly, on Epo expression as well. The collection was prepared by two serial chromatographic steps starting from 10,000 L of HF derived from patients with renal failure and representing a comprehensive source of blood peptides with a molecular weight lower than ~30 kDa. Subjects affected by end stage renal disease (ESRD) require regular haemofiltration in order to eliminate toxic substances without loss of large plasma proteins, immunoglobulin and blood cells, a function normally exerted by the kidneys. Moreover, ESRD patients display inappropriately low Epo levels, despite the high degree of anaemia (Caro et al., 1979): it is likely that Epo regulatory peptides are enriched in HFs from patients with renal failure.

Briefly, HFs are collected (20-30 L/patient/treatment) and subjected to a first cation-exchange chromatography followed by elution with 8 buffers with different pH (from acidic pH 3.6 to basic pH 9.0). Each pH pool is subsequently run over a reverse-phase chromatography column and 48 fractions are retrieved from each pool. The resulting 384 fractions are lyophilized and stored at -20°C (Schulz-Knappe et al., 1997; Mark et al., 1999). Once an active fraction is identified,

further sub-fractionation steps are required in order to analyse the amino acid sequence by mass spectrometry and determine the identity of the peptide. Similar collections have been successfully screened for other purposes and endogenous bioactive peptides have been identified, such as anti-viral peptides or orphan-receptor ligands (Meder et al., 2003; Fricke et al., 2005; Münch et al., 2007; Borst et al., 2013).

Choosing an appropriate cell model and protocol to perform the screening turned out to be a major challenge, since the concentration of the peptides, as well as their half-life and many other properties, were unknown in the extremely complex fractions. We thus started by transiently transfecting exREPCs with the previously described hypoxia-reporter vector pH3SVL (Wanner et al., 2000) together with an SV40-driven *Renilla* (*RL*) luciferase (pRL-SV40) as a control for transfection efficiency and non-HIF related effects (depicted in figure 8A). In order to choose the most optimal stimulus to induce HIF-reporter activity, we exposed exREPCs to hypoxia and to different hypoxia-mimetics: 8 h at 0.2% O_2 were sufficient to obtain strong and reproducible induction of luciferase counts (figure 8C). We therefore performed the screening of the complete collection, twice, following the protocol scheme in figure 8B: cells were transiently transfected in 96-well plates with the reporter constructs, the next day medium was replaced by non-FCS containing medium (to avoid potential background effects of the serum) and peptide fractions were added every 2 h in normoxia and hypoxia, for a total exposure of 8 h. Results of this first screening round are shown in figure 9: the regression analysis of the ratio firefly (FF)/*RL* in hypoxia (figure 9A) indicated poor reproducibility of the two replicates and inhibitory effect of the majority of the fractions tested. Nevertheless, some fractions displayed reproducible activity, either increasing or decreasing luciferase counts compared to the internal control. We continued with the most promising fractions, #04.24 and #06.09 (figure 9B), and tested several sub-fractions obtained by reverse-phase chromatography from the original fractions. Unfortunately, we experienced problems with the transient transfection approach, in terms of luciferase hypoxic inducibility and transfection reproducibility (data not shown). In order to circumvent these technical complications we decided to change the experimental strategy and repeat the screening in stably transfected Hep3B cells.

The aim of this sub-project was to find peptides that influence the HIF pathway and/or Epo production. We thus studied the hypoxia inducibility of different newly generated stable reporter cell lines, expressing FF luciferase under the control of the human prolyl-4-hydroxylase domain 2 (*PHD2*) promoter (an established HIF target gene (Wollenick et al., 2012)) or of Epo 5'-HRE in combination with SV40 promoter (see chapter 3). We compared the new cell models, generated by LV transduction of Hep3B, to cells transiently or stably transfected with pH3SVL and pRL-

SV40 (all the vectors are depicted in figure 10A). We confirmed FF induction of transiently transfected Hep3B after 8 h of hypoxia, as in the first screening round; none of the other cell models showed increased luciferase counts after 8 h of stimulation. After 24 h of hypoxic exposure, all the reporter cell lines displayed induction of FF activity, albeit to a different extent (figure 10B). The main problem experienced with the novel *PHD2* promoter and Epo 5'-HRE constructs was hypoxic induction of the *RL* gene, which we used to normalize the assay. As shown in figure 10A, these two transfer vectors contain the non-hypoxia responsive *RL* gene closely downstream of the hypoxia-induced FF gene. The distance is probably not long enough to insulate the two expressing cassettes, thus *RL* activity pattern follows the FF one, blunting the hypoxic induction when the ratio FF/*RL* is calculated. However, Hep3B stably transfected with pH3SVL and pRL-SV40 did show reproducible FF hypoxic induction after 24 h at 0.2% O_2, stable *RL* expression and low variability between replicates: we chose this cell model to perform the second round of peptide collection screening. We adjusted the screening protocol as shown in figure 10C: exposure to hypoxia was increased to 24 h and peptide fractions were added every 2 h for the last 8 h of hypoxia. Moreover, 3 different fraction concentrations were added to the cells, in order to check for potential dose-dependent effects. Indeed, the new protocol and cell model gave more reproducible results, as demonstrated by the regression analysis of the fold induction hypoxia/normoxia in figure 11A. Fractions were more broadly distributed between "activatory" and "inhibitory" and we selected 8 total fractions based on the extent of the effect, degree of reproducibility and dose-dependency. In addition, neighbouring fractions were preferentially chosen since the same peptide can be sequentially eluted during chromatography (data concerning selected fractions are shown in figure 11B). We are currently confirming these results by treating the samples with the aspecific protease subtilisin or with heat inactivation: these treatments will validate if the effect observed is dependent on the peptide component of the fractions. Based on the results obtained in the confirmation phase, selected fractions will be subjected to sub-fractionation steps in order to identify the HIF regulating peptides.

Discussion

A number of experimental observations in human and animals suggest that *EPO* transcription could be regulated by circulating peptides, albeit no humoral factor has been demonstrated so far to act directly on Epo-producing cells. In the first part of this project, we selected and tested *in vitro* a series of candidate peptides for which a link with Epo production has been indicated. Based on our results, we concluded that none of the candidates had a significant effect on Epo

synthesis in Hep3B cells under our experimental conditions. This could be explained by the fact that renal Epo-producing cells (REPCs), the main source of Epo in adults, are most likely differentially sensitive to stimulation with the humoral factors of interest compared to Hep3B. We evaluated expression of the receptor mRNA in our liver-derived cell model before treating the cells with the corresponding stimulus, but expression levels as well as activation status could differ between Hep3B and REPCs. Immunohistochemistry or immunofluorescence analysis of renal receptor expression *in vivo* are required in order to choose the most promising candidates to treat animals with before checking Epo levels in normoxia and hypoxia. Another possible approach consists in overexpressing *in vitro* the receptor of interest prior to treatment with the hormone. We carried out this strategy by overexpressing ERα in Hep3B (figure 7) but the transfection efficiency was probably too low to see an effect on endogenous transcription, as demonstrated by the GFP control. Concerning the effect of E2 on HIF-2α mRNA, it is possible that Hep3B do not express the right set of receptors/signalling machine to respond to estrogen treatment like MCF-7, a breast cancer-derived cell line which express ERα, ERβ and PgR at considerable levels and show estrogen-dependent growth (Levenson and Jordan, 1997). Generation of stable receptor-overexpressing cell lines and optimization of hormone treatment would be necessary to draw conclusions on the effect of the tested candidates on *EPO* transcription.

Since treatment of Hep3B with specific putative Epo-regulators gave inconclusive results, we decided to complement our investigation with a different approach: we sought HIF-regulating factors in a comprehensive patient HF-derived peptide collection. This strategy has the advantage to be *a priori*, thus allowing identification of unknown peptides as well, and to use endogenous material from human subjects affected by ESRD, thereby increasing the probability to find Epo-regulating peptides. Epo response to anaemia is in fact impaired in these patients (see general introduction and results) and production of such Epo regulators is likely to be increased. Despite difficulties encountered with selection of a suitable protocol and reproducibility of the initial screening rounds, stably transfected cell models gave reliable results and some fractions did show an interesting “activatory” or “inhibitory” activity in our HIF-reporter assay (figure 11). Confirmation of the effect and further sub-fractionation steps are required in order to identify single peptides responsible for the effect. It must not be forgotten that the composition of each peptide fraction is extremely complex and sub-fractionation could lead to increased activity, because of dilution of inhibitory factors present in the same fraction, as much as loss of the activity, because of the necessity of more factors to act simultaneously.

Unfortunately, the outcome is not predictable at this level of complexity and test of different sub-fraction sets is ongoing.

A parallel approach we are using to achieve the aim of finding novel Epo-regulatory hormones is represented by the generation of Epo-reporter cell lines. As described in chapter 3, we characterized a previously unknown HRE located upstream of the *EPO* gene. The first Epo 5'-HRE containing construct we generated (figure 10A) showed hypoxic inducibility but contained a strong and artificial viral promoter, the SV40 promoter. Therefore, we built a new construct containing Epo 5'-HRE, the endogenous minimal promoter and the 3'-HRE of the gene, depicted in figure 12. This construct should contain the most relevant *EPO* locus regulatory elements, thus being the most optimal Epo-reporter up to date. Generation of stable cell lines via LV infection using this construct and a TK (thymidine kinase) promoter-driven *RL* as non-hypoxia inducible control is ongoing and will be used for confirmation of fractions selected from the screening in HIF reporter cells, as well as for future screening rounds.

Figure 1

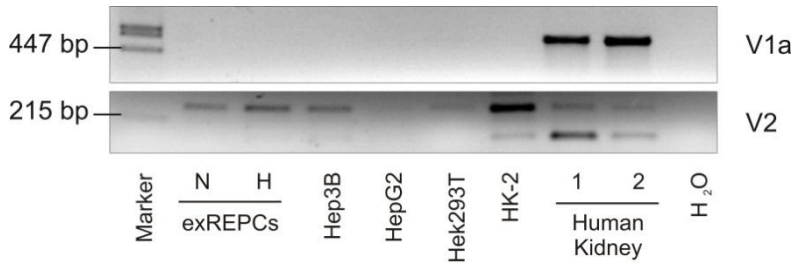

Figure 1 Expression levels of ADH receptors V1a and V2 assessed by RT-PCR using cDNA from different human cell lines. Samples of human kidney and HK-2 cells cDNA were used as positive control. The PCR for V2 receptor results in 2 bands, corresponding to 2 known transcript variants of the gene, one of 215 bp long and one of 148 bp in length. Quality of cDNA was checked by evaluating L28 transcript levels (data not shown).

Figure 2

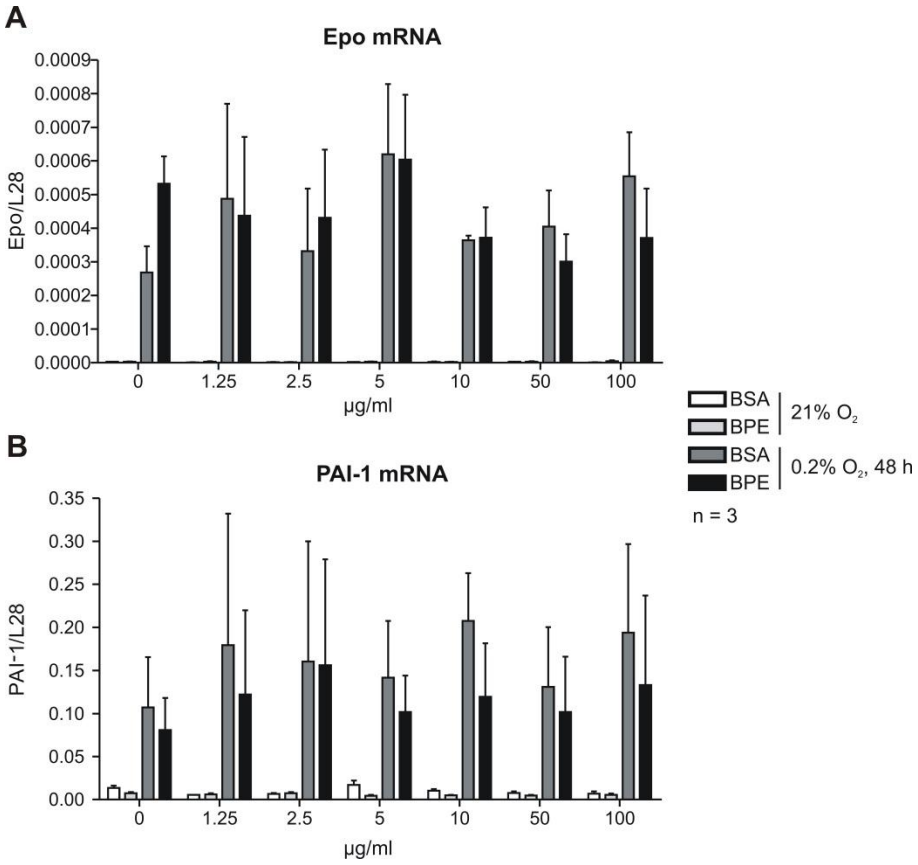

Figure 2 Hep3B cells were treated with different concentrations of BPE or BSA control, for 48 h in normoxia or hypoxia (0.2% O_2). Epo (**A**) and PAI-1 (**B**) mRNA levels were measured by RT-qPCR. All data are expressed as mean ± SEM.

Figure 3

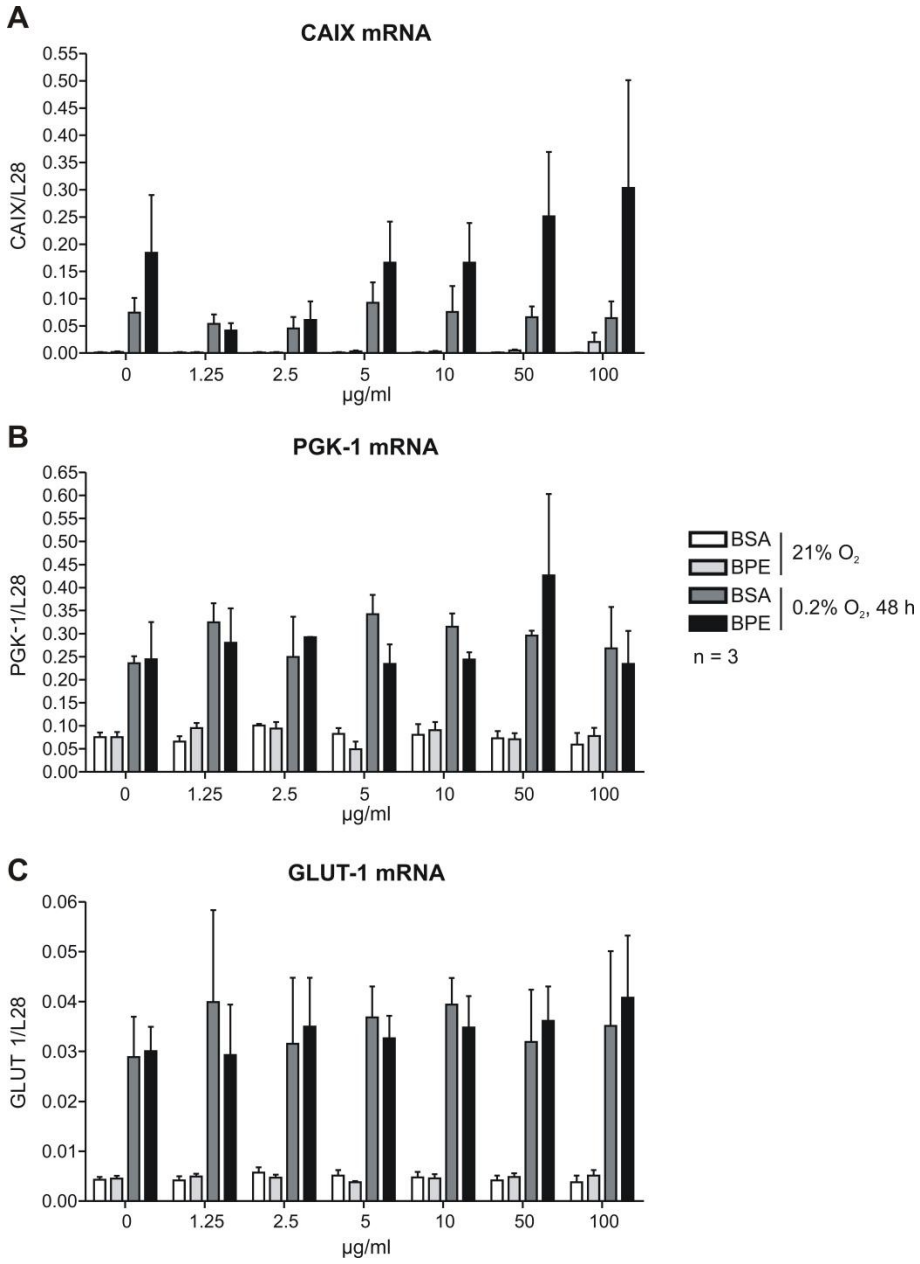

Figure 3 Hep3B cells were treated with different concentrations of BPE or BSA control, for 48 h in normoxia or hypoxia (0.2% O_2). CAIX (**A**), PGK-1 (**B**) and GLUT-1 (**C**) mRNA levels were measured by RT-qPCR. All data are expressed as mean ± SEM.

Figure 4

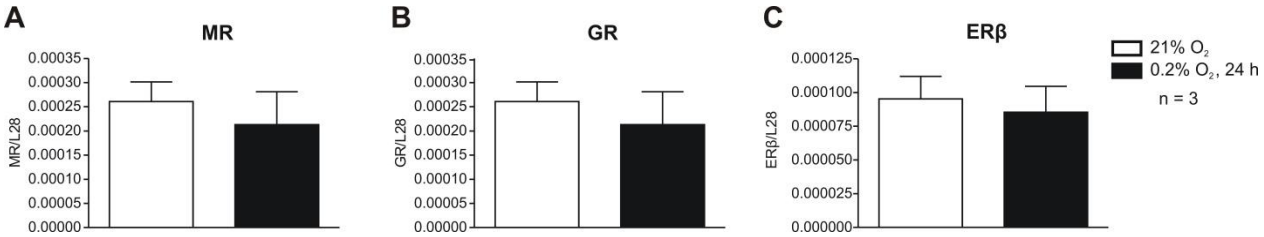

Figure 4 MR (**A**), GR (**B**) and ERβ (**C**) mRNA levels were measured in normoxic and hypoxic Hep3B by RT-qPCR. All data are expressed as mean ± SEM.

Figure 5

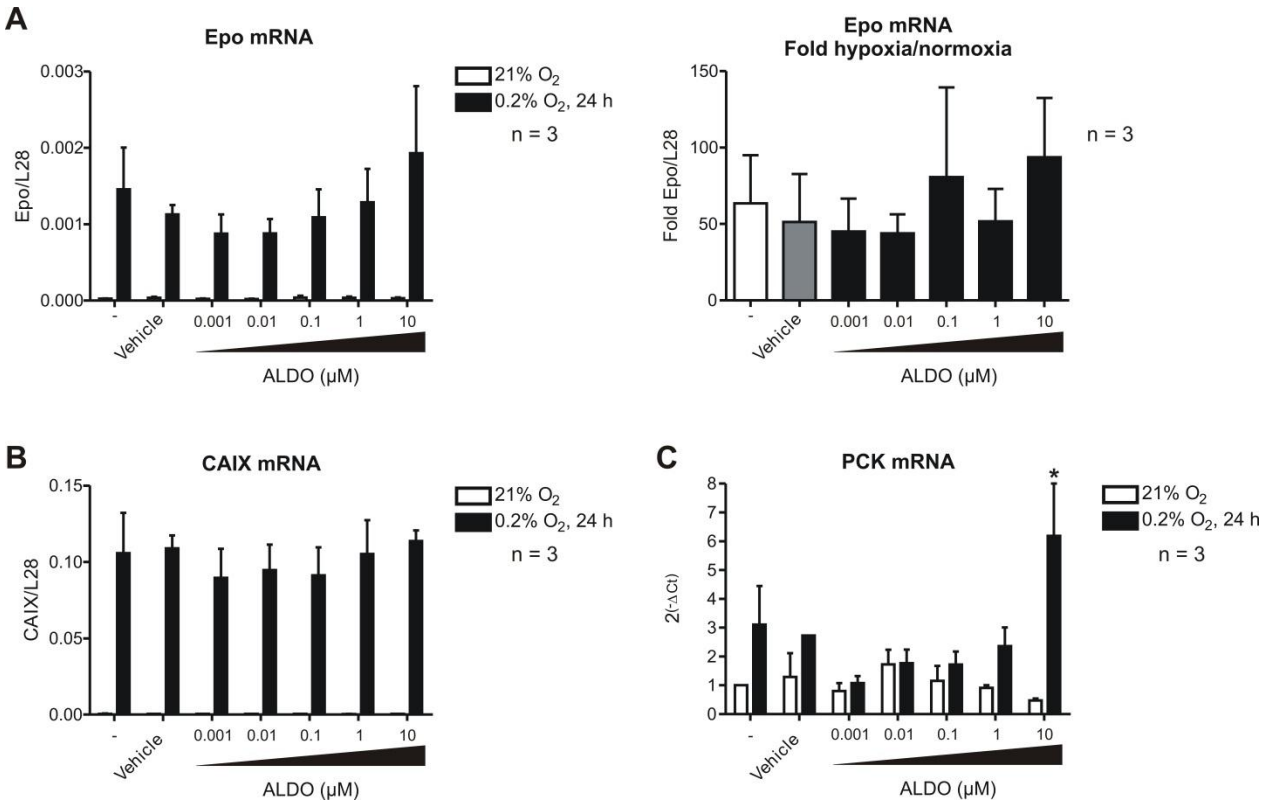

Figure 5 Hep3B cells were treated with different concentrations of ALDO (vehicle control: 0.014% ethanol, corresponding to the amount used for the highest concentration of ALDO), for 24 h in normoxia or hypoxia (0.2% O_2). Epo (**A**), CAIX (**B**) and PCK (**C**) mRNA levels were measured by RT-qPCR. All data are expressed as mean ± SEM. Statistics: t-test, *$p < 0.05$, compared to vehicle control in hypoxia.

Figure 6

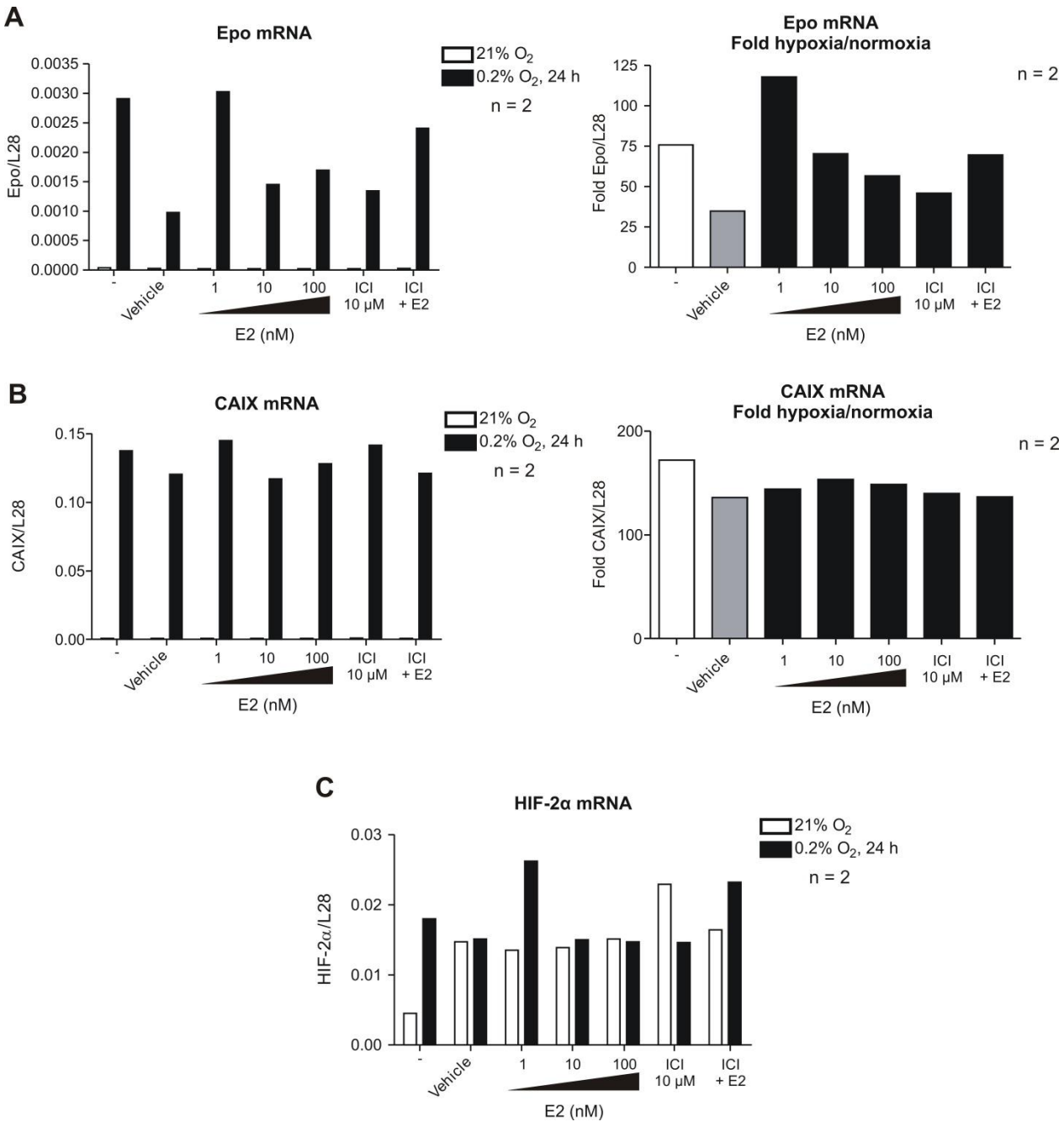

Figure 6 Hep3B cells were treated with different concentrations of E2 and ICI (vehicle control: 0.02% ethanol, corresponding to the amount used for the highest concentration of E2), for 24 h in normoxia or hypoxia (0.2% O_2). In the combined treatment, 10 µM ICI and 10 nM E2 were used. Epo (**A**), CAIX (**B**) and HIF-2α (**C**) mRNA levels were measured by RT-qPCR. All data are expressed as mean alone.

Figure 7

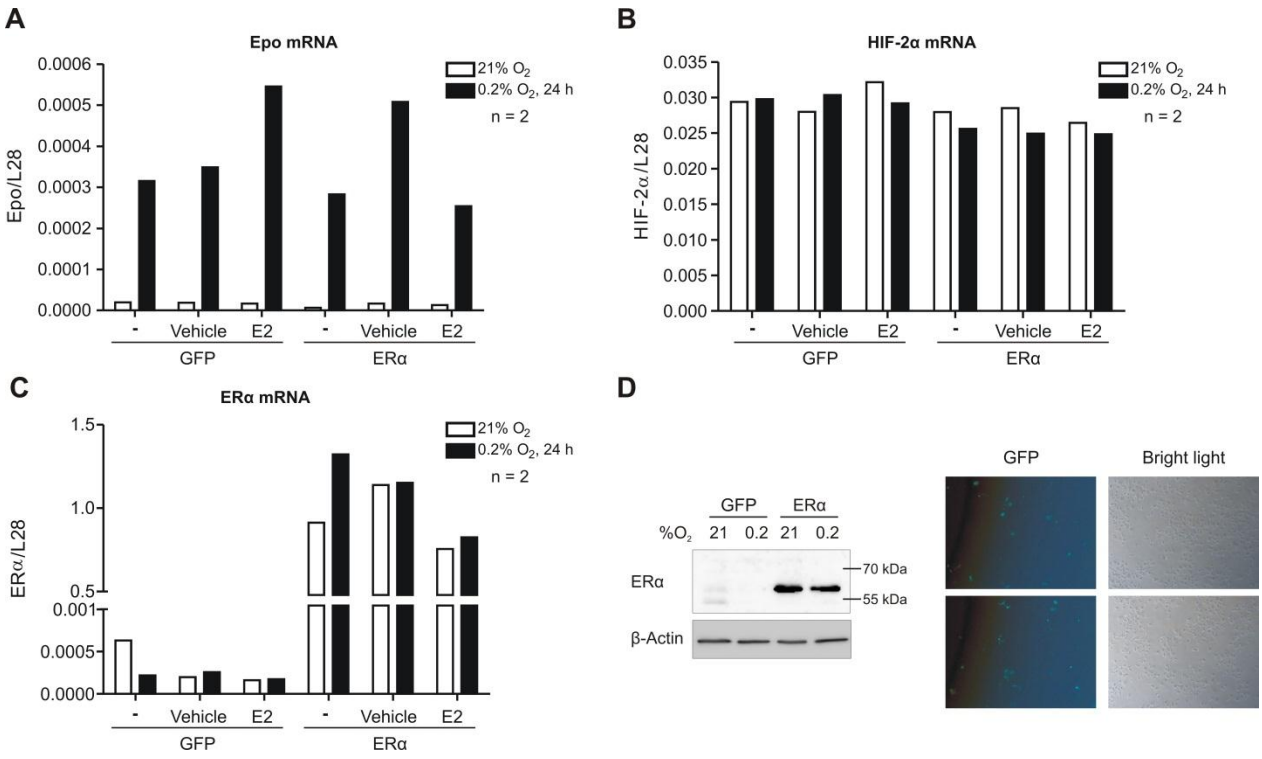

Figure 7 Hep3B cells were transfected with a GFP- or an ERα-overexpressing vector and treated with 10 nM E2 (vehicle control: 0.02% ethanol), for 24 h in normoxia or hypoxia (0.2% O_2). Epo (**A**), HIF-2α (**B**) and ERα (**C**) mRNA levels were measured by RT-qPCR. ERα overexpression was evaluated by immunoblotting as well (**D**, left panel). GFP-transfected cells were used to check efficiency of transfection with a fluorescent microscope (**D**, right panel). All data are expressed as mean alone.

Figure 8

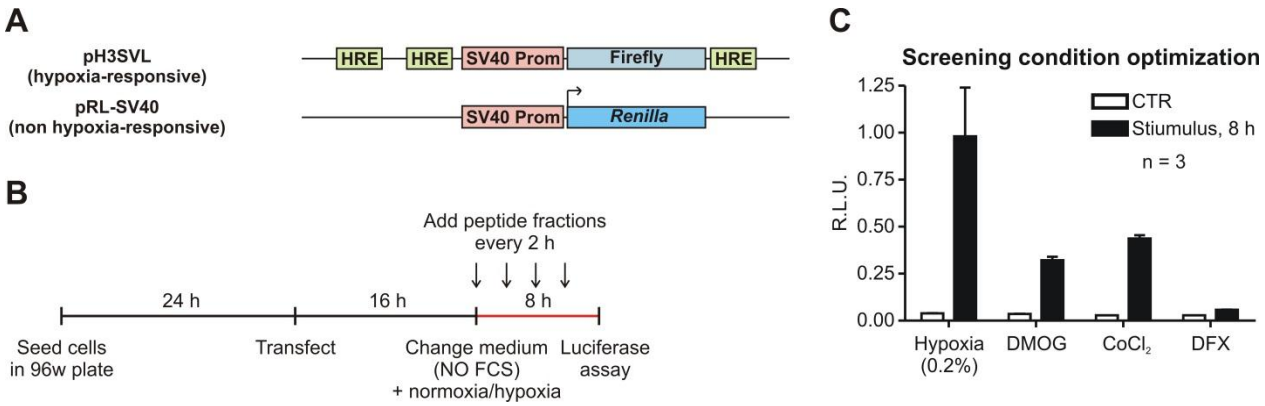

Figure 8 ExREPCs were transiently transfected with pH3SVL and pRL-SV40 (depicted in **A**) and exposed to normoxia, hypoxia or hypoxia mimetics for 8 h; results of the luciferase assay are displayed as R.L.U. (**C**). All data are expressed as mean ± SEM. **B.** schematic representation of the screening protocol.

Figure 9

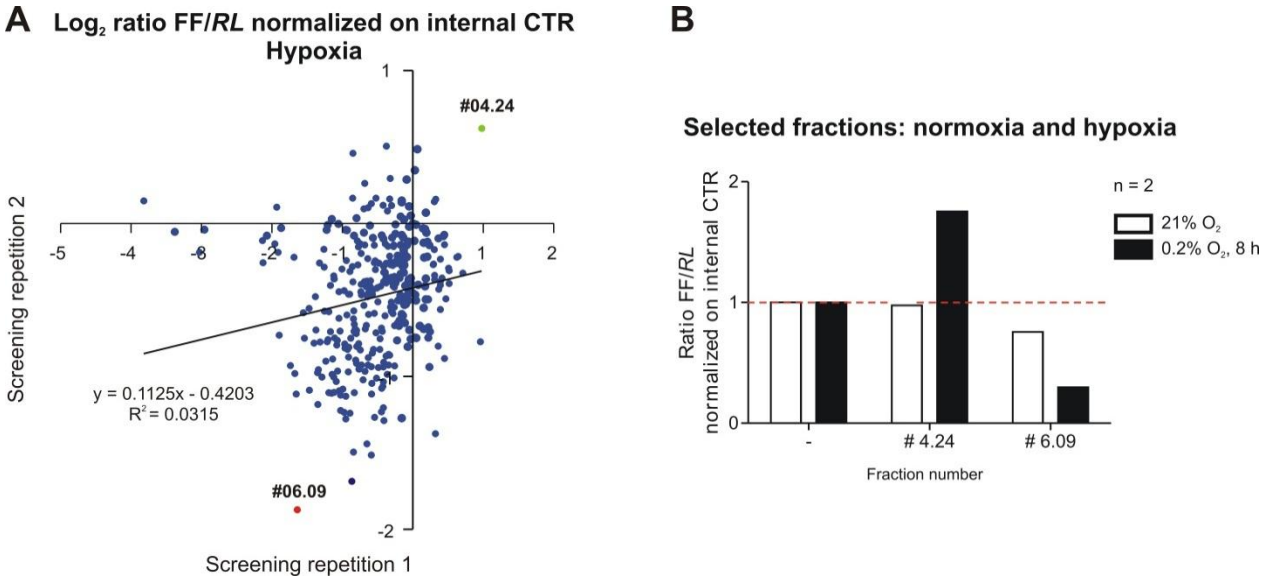

Figure 9 A. Regression analysis of the ratio FF/*RL* in hypoxia normalized on internal control (CTR): each dot represents one of the 384 peptide fractions in the two screening repetitions; the most “activatory” and “inhibitory” fractions are highlighted in green and red, respectively. Axes origin represents the CTR. **B.** Results of the luciferase assay from selected fractions #04.24 and #06.09 are displayed as R.L.U. All data are expressed as mean alone.

Figure 10

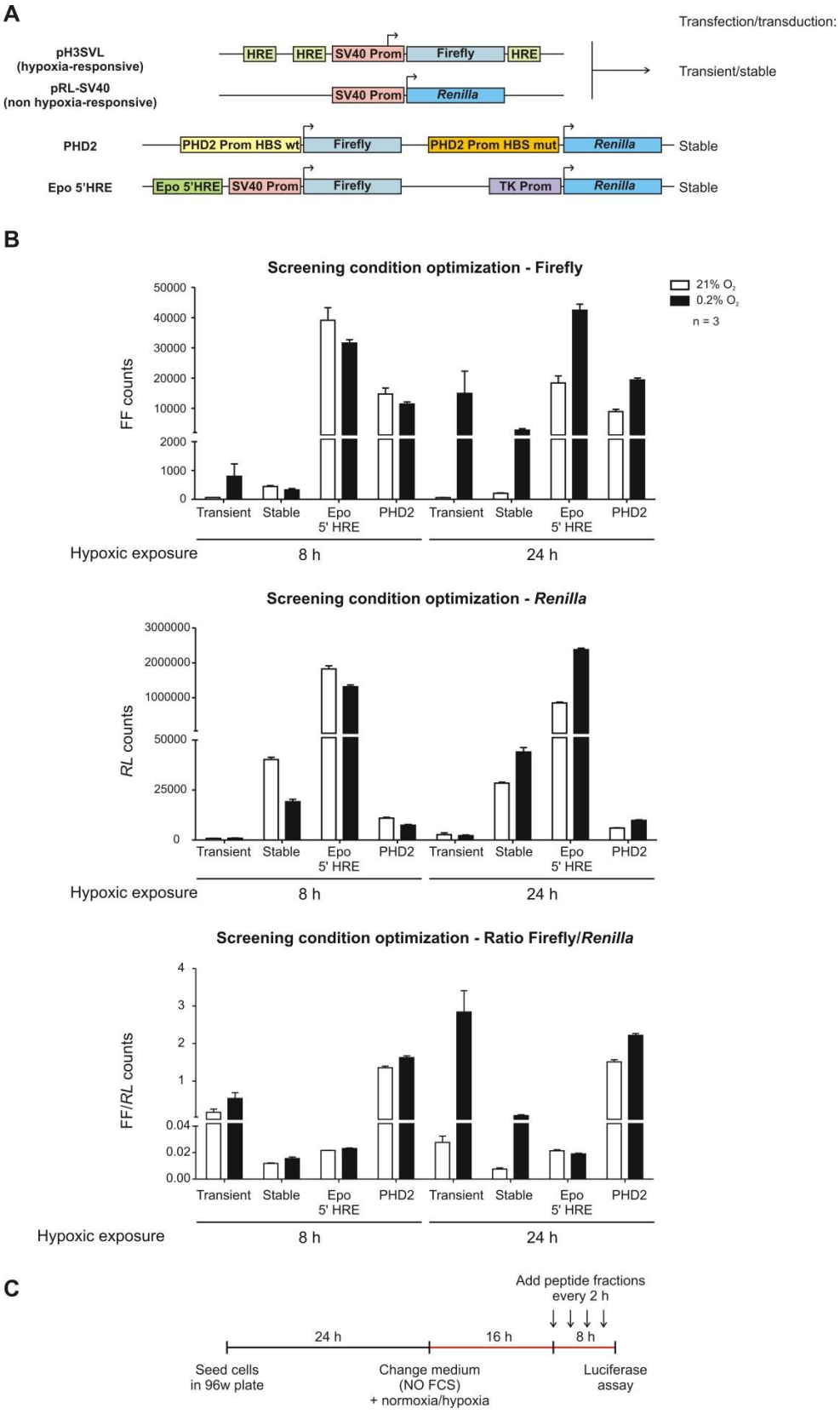
A
Transfection/transduction:
pH3SVL
(hypoxia-responsive)
HRE
HRE
SV40 Prom
Firefly
HRE
Transient/stable
pRL-SV40
(non hypoxia-responsive)
SV40 Prom
Renilla
PHD2
PHD2 Prom HBS wt
Firefly
PHD2 Prom HBS mut
Renilla
Stable
Epo 5'HRE
Epo 5'HRE
SV40 Prom
Firefly
TK Prom
Renilla
Stable
B
Screening condition optimization - Firefly
21% O2
0.2% O2
n = 3
FF counts
50000
40000
30000
20000
10000
2000
1000
0
Transient
Stable
Epo 5' HRE
PHD2
Transient
Stable
Epo 5' HRE
PHD2
Hypoxic exposure
8 h
24 h
Screening condition optimization - Renilla
RL counts
3000000
2000000
1000000
50000
25000
0
Transient
Stable
Epo 5' HRE
PHD2
Transient
Stable
Epo 5' HRE
PHD2
Hypoxic exposure
8 h
24 h
Screening condition optimization - Ratio Firefly/Renilla
FF/RL counts
4
3
2
1
0.04
0.02
0.00
Transient
Stable
Epo 5' HRE
PHD2
Transient
Stable
Epo 5' HRE
PHD2
Hypoxic exposure
8 h
24 h
C
Add peptide fractions every 2 h
24 h
16 h
8 h
Seed cells in 96w plate
Change medium (NO FCS) + normoxia/hypoxia
Luciferase assay

Figure 10 Hep3B were transiently or stably transfected or transduced with pH3SVL, pRL-SV40, *PHD2* promoter or Epo 5'-HRE reporter vectors (depicted in **A**) and exposed to hypoxia for 8 or 24 h; results of the luciferase assay are displayed as R.L.U. (**B**). All data are expressed as mean ± SEM. **C**. schematic representation of the screening protocol.

Figure 11

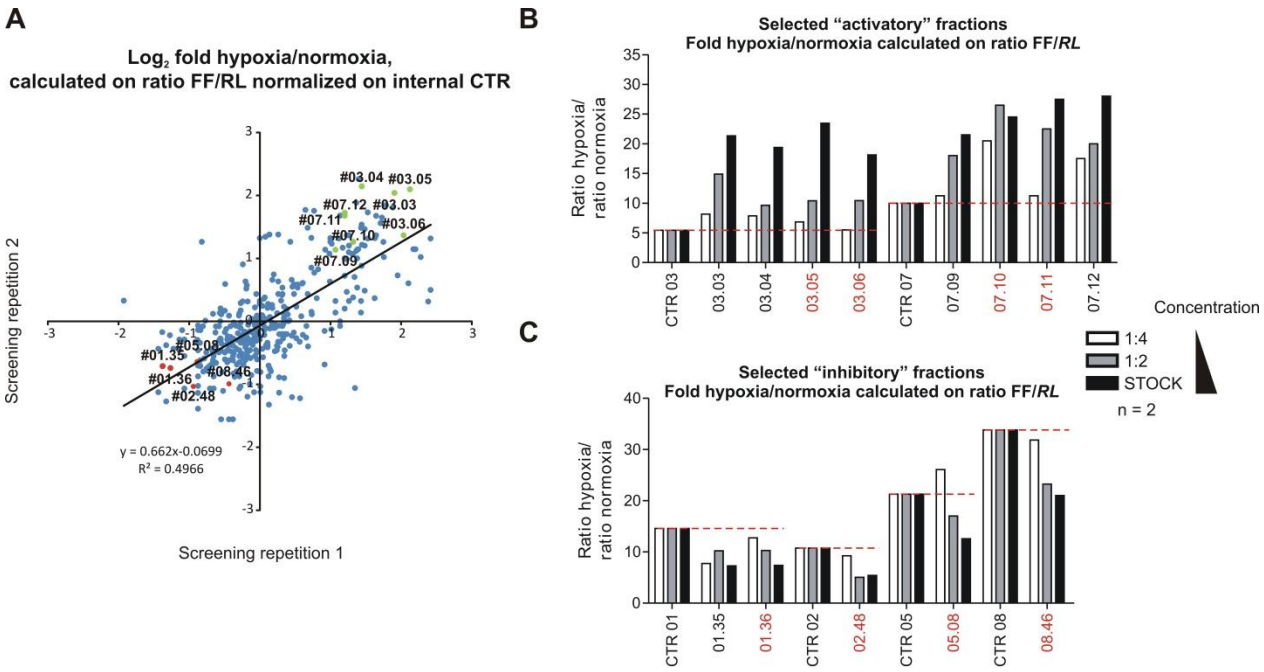

Figure 11 A. Regression analysis of the fold hypoxia/normoxia calculated on ratio FF/*RL* and normalized on internal control (CTR): each dot represents one of the 384 peptide fractions in the two screening repetitions (STOCK concentration); the selected "activatory" and "inhibitory" fractions are highlighted in green and red, respectively. Axes origin represents the CTR. **B.** and **C.** Results of the luciferase assay from the most "activatory" and "inhibitory" fractions, respectively, at different concentrations are displayed as fold induction hypoxia/normoxia. Fractions marked in red were selected for further confirmation and sub-fractionation. Data are expressed as mean alone.

Figure 12

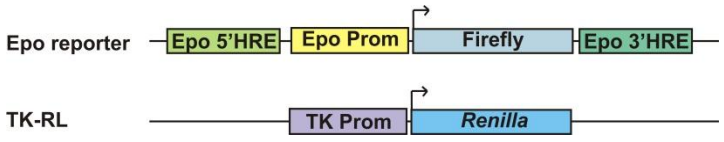

Figure 12 Schematic representation of the novel Epo-reporter and TK-RL vectors. The two constructs were cloned into the transfer vectors pDestX1-puromycin and pDestX1-neomycin, allowing generation of LV particles.

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8. CRISPR/Cas-mediated genome editing to dissect the role of endogenous distal and proximal hypoxia response elements in regulating oxygen-dependent Epo expression

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Introduction

Erythropoietin (Epo) is the key hormone promoting red blood cell production in response to hypoxia and anemia. While the kidney is the primary site of adult Epo synthesis, the liver is the main source during development. Hypoxia-inducible Epo transcription is controlled by distinct regulatory sequences in liver and kidney. These regulatory regions flank the Epo coding sequence on both sides, the long-sought for kidney-inducible element (KIE) located far upstream in the 5′-region and the well-established liver-inducible element in the 3′-region. We recently characterized a novel hypoxia-response element (HRE) located upstream of the erythropoietin (*EPO*) gene, named Epo 5'-HRE and likely representing the hitherto unidentified KIE (Storti et al., 2014). We could show that the functional 5'-HRE is specifically induced by hypoxia-inducible factor (HIF)-2α using reporter gene assays and that endogenous HIF-2α protein binding on the HRE is enriched in hypoxic Hep3B cells. We propose that the Epo 5'-HRE represents the hypoxia-inducible regulatory element necessary for renal inducibility of the gene. The enhancer is indeed located within the so called KIE, as defined by transgenic animal experiments, and the arrangement of the HIF binding site (HBS) and CACA repeat resembles the one of the 3'-HRE, the established Epo liver-inducible element (Köchling et al., 1998; Suzuki et al., 2011). HIF-2 specificity of Epo induction further supports our hypothesis (see introduction, section 1.3). In order to assess the contribution of the novel 5'-HRE in endogenous *EPO* transcription, we aimed to mutate this element in the genomic context of Hep3B, the same established Epo-producing cell line used for functional characterization of the HRE (see chapter 3). Our approach consists in specifically targeting the HBS using a novel genome targeting technique: the cluster regularly interspace short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) technology.

Targeted genome editing, i.e. modification of the endogenous genomic DNA (gDNA) sequence at precise, predetermined loci, has been extensively used in biomedical research to study the function of genes, regulatory elements, disease-causing mutations and polymorphisms (Kim and

Kim, 2014). The classic approach, used for instance to generate knock-out and knock-in animals, consists in recombination of the genomic locus of interest with a homologous cassette, resulting in interruption of the gene or replacement with the desired mutation. Homologous recombination strategies in mouse embryonic stem cells or other mammalian cells allowed in the past decades generation of sophisticated animal and cell models, including conditional knock-out/knock-in with help of enzymes like Cre and FLP recombinases (reviewed by (Capecchi, 2005)). Nevertheless, classic genome editing remains complex and time-consuming. Nowadays more efficient strategies based on the use of site-specific nucleases, enzymes that cleave double strand (ds)DNA at the desired position, are broadly used to target gDNA loci in mammalian cells and include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas technology. These techniques mainly differ in the way by which precise genome targeting is achieved: TALENs and ZFNs are based on modular proteins designed to bind specific DNA sequences, while the CRISPR/Cas system relies on complementary RNA-DNA hybridization to guide the nuclease at the desired site in the genome.

ZFNs are recombinant proteins consisting of a FokI restriction enzyme nuclease domain at the C-terminus and a series of zinc-finger DNA-binding domains at the N-terminus, each recognizing a 3 bp DNA sequence. Target specificity is achieved by generating recombinant, modular zing-finger proteins (Gupta et al., 2012; Bhakta et al., 2013). Not all the combinations of DNA-binding motives, however, lead to efficient cleavage: it has been calculated that a single functional ZFN pair can be obtained on average every ~100-bp DNA sequence (Kim et al., 2009). A similar modular approach is valid for TALENs, where the FokI nuclease domain is fused to DNA binding motives derived from the plant pathogenic bacterium *Xanthomonas*. Each domain recognizes a specific nucleotide, thus allowing generation of modular proteins able to recognize virtually any DNA sequence (Moscou and Bogdanove, 2009; Deng et al., 2012). The only requirement seems to be the presence of a thymine at the 5' end of the target sequence, although contradictory data on the stringency of this requirement can be found in literature (Kim and Kim, 2014). Since the FokI cleavage domain needs dimerization to generate double strand breaks (DSBs), both TALENs and ZFNs are used in pairs, simultaneously increasing target sequence length and specificity. Two monomers are designed to bind adjacent half-sites that are separated by a 5-7 bp spacer for ZFNs and a 12-14 bp spacer for TALENs (Bitinaite et al., 1998). Cells are then transfected with two plasmids, each overexpressing one of the specific nucleases, and single cell-derived colonies are screened for efficient genome editing. Compared to ZFNs, the TALEN technology shows improved targeting efficiency, decreased cytotoxicity and decreased off-target

effects but still requires the design of two recombinant proteins and a target sequence size of approximately 30-40 bp to reach efficient and specific genome editing (see table 1).

The novel CRISPR/Cas system uses RNA-guided engineered nucleases (RGENs) and is derived from bacterial and archaea adaptive immune system. In early 2013, four research teams reported engineering of the bacterial system in order to target mammalian cell genome and in the past two years a rapidly increasing number of publications have exploited the CRISPR/Cas system to generate desired mutations in human cells, mice, rats, zebrafish, bacteria, Drosophila, yeast and nematodes (Pennisi, 2013). This technology uses guiding (g)-RNAs that are complementary to the desired gDNA locus to guide the Cas9 endonuclease, capable of generating DSBs at the position of interest. The most used system so far is called type II and is derived from *Streptococcus pyrogens*. In the natural pathway, the CRISPR operon in the bacterial genome contains all the genes necessary for functionality of the system, including different Cas genes and a long pre-CRISPR (pre-cr)-RNA. gRNAs are generated from processing of the long non-coding pre-crRNA, which consists of short palindromic repeats interspaced by variable sequences, the proto-spacers. The latter represent the adaptive part of the defence mechanism since they are complementary to exogenous DNA potentially derived from phages or other pathogens. An auxiliary trans-activating crRNA (tracrRNA), transcribed from the same operon, helps the processing of long precursors by RNase III into discrete units containing a 20 nucleotide (nt) gRNA and a partial direct repeat, generally forming a hairpin structure and interacting with Cas9. In the type II CRISPR system the target sequence, complementary to the gRNA, must immediately precede a 5'-NGG-3' proto-spacer adjacent motif (PAM, where N can be any nt). The PAM sequence is probably used for selection of proto-spacers in invading nucleic acid to be included in the CRISPR operon. It is in fact required, together with the gRNA, for specific binding of Cas9 to target DNA, resulting in cleavage of the sequence (Makarova et al., 2011). An overview of the endogenous type II CRISPR/Cas system is depicted in figure 1.

	ZFNs	TALENs	RGENs
DNA targeting specificity determinant	Zinc-finger proteins	Transcription activator-like effectors	crRNA or sgRNA
Nuclease	*Fok*I	*Fok*I	Cas9
Success rate‡	Low (~24%)	High (>99%)	High (~90%)
Average mutation rate§	Low or variable (~10%)	High (~20%)	High (~20%)
Specificity-determining length of target site	18–36 bp	30–40 bp	22 bp (total length 23 bp)
Restriction in target site	G-rich	Start with T and end with A (owing to the heterodimer structure)	End with an NGG or NAG (lower activity) sequence (that is, PAM)
Design density	One per ~100 bp	At least one per base pair	One per 8 bp (NGG PAM) or 4 bp (NGG and NAG PAM)
Off-target effects	High	Low	Variable
Cytotoxicity	Variable to high	Low	Low
Size	~1 kb×2	~3 kb×2	4.2 kb (Cas9 from *Streptococcus pyogenes*) + 0.1 kb (sgRNA)

Cas9, CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 9; crRNA, CRISPR RNA; N, any nucleotide; PAM, protospacer adjacent motif; RGEN, RNA-guided engineered nuclease; sgRNA, single-chain guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease. *A wide range of success rates and mutation rates (which depend on factors such as the methods used to construct these nucleases, delivery methods and cell lines or organisms) have been reported. The numbers given here are based on our own studies using HEK293 cells[5,15,54,62,124,192]. Mutation frequencies are higher in K562 cells and HeLa cells than in HEK293 cells. ‡The success rate is defined as the proportion of nucleases that induce mutations at frequencies >0.5% in HEK293 cells. §The average mutation rate is based on the frequency of non-homologous end-joining-mediated insertions and deletions obtained at the nuclease target site.

Table 1 Comparison between the available nuclease-based genome editing technologies (Kim and Kim, 2014).

For *in vitro* and *in vivo* genome editing purposes, the crRNA and tracrRNA have been combined into a single guiding (sg)-RNA containing the sequence complementary to the 20 nt target of interest, followed in the genomic sequence by the PAM. The sgRNA-expressing vector is co-transfected with a codon-optimized Cas9 expressing vector and single colonies are screened for targeting of the desired locus. It has been calculated that 5'-NGG-3' sequences are found in the human genome every 8-12 bp, thus making this system less versatile compared to TALENs but much easier to design (Ran et al., 2013). Moreover, ZFNs and TALENs cut randomly within the spacer sequence between the two adjacent half sites, whereas Cas9 cleaves exactly between the 17th and 18th position in the target sequence (3 bp upstream of the PAM) (Jinek et al., 2012).

For all the genome editing techniques described, repair of the DSBs can follow the same two major DNA-repair pathways: the error-prone non-homologous end joining (NHEJ) or the high fidelity homology-directed repair (HDR, figure 2). NHEJ generates random mutation in the form of small insertion/deletion around the cleaved site and can result in frame-shift or premature stop-codon formation in coding regions. When a single or double strand DNA template is provided, HDR mediate homologous recombination of the DSB, allowing insertion of the exact desired sequence. However, HDR is usually less frequent than NHEJ and active only in dividing cells (Saleh-Gohari and Helleday, 2004; Chen et al., 2011).

One of the advantages of RGENs compared to ZFNs and TALENs is the possibility of efficient and simultaneous targeting of different genomic loci, therefore generating cells or animals with multiple gene mutations. Despite generation of rats with double mutations by zygote injection of two ZFN pairs and corresponding HDR dsDNA templates was recently reported (Brown et al., 2013), the complex and time-consuming design and production of ZFNs and double-stranded donor vectors limit the application of this method. The CRISPR/Cas technology has instead been used for one-step generation of mice with up to 5 simultaneous genome modifications (Wang et al., 2013; Yang et al., 2013; Zhou et al., 2014) and a vector containing multiple sgRNAs together with Cas9 was successfully used to target multiple loci in human cells with a single transfection (Sakuma et al., 2014). On the other hand, frequency of off-targets seems to be higher with RGENs compared to the other site-directed nucleases. Cas9 can tolerate mismatches between the gRNA and the DNA target sequence, especially in the 5' region of the RNA (opposite to the PAM), thus cutting homologous DNA loci as well (Fu et al., 2013; Lin et al., 2014). Other reports in literature show contradictory data and describe substantially low frequency of off-targets for the CRISPR/Cas system in mammalian cells (Cho et al., 2014; Veres et al., 2014). Based on genome-wide analysis of Cas9 binding in mouse embryonic stem cells, it is likely that Cas9 has many off-target binding sites but actually cleaves only a small fraction of them (Wu et al., 2014). New strategies to decrease the possibility of off-targets include generation of informatics tools for improved design of gRNA sequences, optimization of extent and duration of Cas9 expression and use of paired Cas9 nickases instead of nucleases. Nickase enzymes are known to have low off-target activity and generate single strand breaks (SSBs) in the DNA locus: if the two SSBs are close enough, the NHEJ machine will repair the damage, thus resulting in the same effect of a DSB (Cho et al., 2014; Shen et al., 2014).

Given the high success rate of the CRISPR/Cas technique in mammalian culture and the easy design of specific sgRNA sequences, we decided to exploit the advantages of this novel system in order to separately mutate endogenous Epo 5'- and 3'-HRE in Hep3B to assess the relative contribution of these mutations on hypoxia-inducible *EPO* transcription.

Materials and methods

Cell culture

Hep3B, HepG2 and Hek293T were cultured in DMEM (high glucose, Sigma-Aldrich-Aldrich) supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 IU/ml penicillin and 50

µg/ml streptomycin (Invitrogen). Hypoxia experiments were carried out at the indicated concentration of oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400, Ruskinn Technologies).

Generation of stably Epo 5'- or 3'-HRE targeted Hep3B by CRISRP/Cas-mediated genome editing

Cells were plated at a density of $1.5x10^6$ cells per 10 cm dish and transfected with pRGEN-Cas9-HA-CMV, pRGEN-U6-sgRNA for Epo 5'- or 3'-HRE targeting, a 100 bp long single strand (ss)-DNA oligo as a template for HDR and a linearized puromycin resistance vector (3 µg of total DNA) using polyethylenimine (PEI) transfection. pRGEN vectors were all purchased from ToolGen and ssDNA oligos from Microsynth (see figure 3 for sgRNA and partial ssDNA oligo sequences). Control cells were transfected with pEGFP-C1 (Clontech) and a linearized puromycin resistance vector (3 µg of total DNA). 72 h post-transfection, medium was changed to 1.5 µg/ml (Hep3B) or 1 µg/ml (HepG2) puromycin-containing medium for an additional 72 h, until all the wt control cells died. After two weeks of recovery, cells were sub-cloned by limited dilution in 96-well plates or 10 cm dishes and single cell-derived colonies were picked and grown for further experiments.

gDNA extraction and detection of targeted loci

Approximately $1x10^6$ cells were harvested, washed 2x with phosphate-buffered saline (PBS) and re-suspended in 600 µl of 200 mM NaCl, 50 mM Tris/HCl (pH 8.00), 10 mM EDTA and 1% sodium dodecyl sulphate (SDS) lysis buffer. Proteinase K (15 µl, Thermo Scientific) was added and lysates were incubated at 65°C overnight. On the next day, 200 µl of water-saturated NaCl was added, followed by precipitation at 15 krpm for 30 minutes at 4°C. Supernatants (750 µl) were transferred to a new tube and DNA was precipitated with 450 µl of isopropanol at 15 krpm for 30 minutes at 4°C. DNA pellet were washed with 70% ethanol, air-dried and re-suspended in 50 µl of Tris/EDTA (TE) buffer.

500 ng of gDNA were used as template for PCR amplification of the loci of interest using the primers and annealing temperatures listed in table 2. Primer pairs (2) contain XhoI restriction sites (in bold) for cloning into pBlueScriptII SK (+) and sequencing (see below). PCR products were evenly split for overnight digestion with TaiI or PstI (Thermo Scientific) at 65/37°C and loaded on a 2.1% agarose gel.

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (TaiI/PstI restriction)
5'-HRE (1)	CCACATCCTTTCCATCAGCCT	GGAACTGCCAAAACAAACCAGAT	505 bp (108/73 bp)
5'-HRE (2)	CCCC**CTCGAG**TCAGTGGCAATGTGGAG	CCCC**CTCGAG**CTGCGGACATTTCTATCA	148 bp (195/133 bp)
3'-HRE (1)	TGGCAAGCTGTGACTTCTCC	GCCCTGGGCAGGGTTG	198 bp (301/132 bp)
3'-HRE (2)	CCCC**CTCGAG**TGGCAAGCTGTGACTTCT	CCCC**CTCGAG**CCCTGGGCAGGGTTG	362 bp (304/140 bp)

Table 2 Primers used for gDNA amplification

gDNA loci sequencing

500 ng of gDNA from selected colonies were used as template for PCR amplification of the loci of interest using XhoI restriction site-containing primers (table 2). After overnight digestion with XhoI, PCR products were cloned into pBlueScriptII SK (+) vector and sequenced with a M13 primer.

RNA extraction and quantitative PCR

RNA was extracted by using the phenol-chloroform method and cDNA was generated by reverse transcription (RT) of 2 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were quantified by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma-Aldrich) in combination with a MX3000P light cycler (Agilent) and the primers listed in table 3. Initial template concentrations were calculated by comparison with serial dilutions of a calibrated standard. Ribosomal protein L28 mRNA levels were used to normalize the data.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size
hL28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198 bp
hEpo	TCACTGTCCCAGACACCAAA	CCTCCCCTGTGTACAGCTTC	362 bp
hCITED-2	GGAGCAGAAATCGCAAAAAC	GACCCATGAACTGGGAGTTG	334 bp
hCAIX	GGGTGTCATCTGGACTGTGTT	CTTCTGTGCTGCCTTCTCATC	309 bp
hPGK-1	AACAAGGTTAAAGCCGAGCC	TGAGCTGGATCTTGTCTGCA	259 bp

Table 3 Primers used for qPCR amplification

Immunoblotting

Combined cytoplasmic and nuclear extracts were prepared using a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 x protease inhibitory cocktail (Sigma-Aldrich). Protein concentrations were determined by the bicinchoninic acid assay method and 40 µg of cellular protein was subjected to immunoblot analyses. Membranes were probed with antibodies against HIF-1α (clone 54/HIF-1α, BD Transduction Laboratories), HIF-2α (PAB12124, Abnova) and β-actin (Sigma-Aldrich). Signals from HRP-coupled secondary antibodies were detected with ECL substrate (all Pierce) using a luminescent image analyser (LAS-4000, FUJIFILM). β-actin was used as loading control for combined cytoplasmic and nuclear extracts.

Results

In order to achieve efficient targeting of the Epo 5'- and 3'-HRE in Hep3B, we first optimized several transfection conditions, including amount of cells, amount of DNA, transfection reagent and puromycin concentration. gDNA was extracted from transfected pools of cells before and after puromycin selection and efficiency of the targeting was assessed by restriction analysis of the loci of interest, following the scheme depicted in figure 3. The PCR amplicon from wt gDNA is completely digested by TaiI, whereas efficient HRE targeting destroys the TaiI restriction site, resulting in the presence of an undigested band on the gel. Moreover, when the DSB is repaired via HDR, using a 100 bp ssDNA oligo as a template, a PstI restriction site is introduced. This strategy easily allows identification of targeted as well as homology recombined alleles. As expected, puromycin selection greatly increased the amount of HRE-mutated amplicons, often not detectable before selection (figure 4). HDR is known to occur less frequently compared to NHEJ (Saleh-Gohari and Helleday, 2004): consistently, we seldom observed PstI-digested bands in the transfected pools. After selecting the most efficiently targeted pools, we continued with single cell cloning in order to obtain clonal populations of Hep3B carrying the same mutated alleles.

Screening of several single cell-derived Hep3B clones revealed that targeting efficiency of the 3'-HRE genomic locus was higher compared to the 5'-HRE one. In order to increase targeting efficiency for the Epo 5'-HRE locus, we transfected the cells twice with the same CRISPR/Cas set of vectors, resulting in slightly higher number of partially targeted colonies (figure 5). Nevertheless, no colony carrying only mutated alleles could be obtained with this approach. A

number of factors can explain the difference between 5'- and 3'-HRE targeting, including different efficiency of the gRNAs in driving the Cas9 on the target sequence and/or chromatin accessibility of the genomic region. Indeed, it was recently shown that the presence of DNase I hypersensitivity sites (DHS) is the strongest predictor of Cas9 binding in a genome-wide analysis performed in mammalian cells (Wu et al., 2014). The HBS contains a CG dinucleotide that can be methylated (Yin and Blanchard, 2000) and CpG methylation was shown by the same study to represent a negative determinant of Cas9 binding as well, even though contradictory results have been reported (Hsu et al., 2013; Wu et al., 2014). To further investigate the possible reason for less efficient targeting of the Epo 5'- compared to 3'-HRE locus, we sequenced the PCR product from both genomic loci in wt cells and compared it to the reference sequence obtained from the UCSC Genome Browser which was used to design the gRNAs. No differences to the UCSC reference sequence could be detected for the 5'- and 3'-HRE PCR products from gDNA derived of wt Hep3B, HepG2 and Hek293T (data not shown). These results exclude impaired hybridization of the gRNA to the target sequence as the reason for lower efficiency of 5'-HRE targeting. As an additional approach to mutate the endogenous 5'-HRE in Epo-expressing cells, we transfected the same set of targeting vectors in HepG2. Figures 5 and 6 show higher success rate in this cell line, with 4 100% mutated clones and several partially mutated ones. Characterization of the 5'-HRE mutated HepG2 clones is ongoing.

In contrast to targeting the 5'-HRE, Hep3B clones with successful CRISPR-Cas-mediated mutation of the 3'-HRE were obtained. Two independent clones were characterized by comparing Epo mRNA levels between these clones and wt cells, the pool of cells transfected with the CRISPR/Cas vectors after puromycin selection and one partially targeted (+/-) clone, which underwent the same single cell cloning procedure as the positive ones. Figure 6B shows measurement of Epo mRNA in normoxic and hypoxic conditions: Epo hypoxic induction was completely abrogated in the two positive, mutated clones (genotype was confirmed in figure 7A). Interestingly, the pool and the partially targeted clone, showing the same genotype, displayed reduced Epo mRNA levels compared to wt cells upon hypoxia, suggesting a dose-dependency of functional HREs and Epo induction. In order to ensure functionality of the HIF pathway, we evaluated the behaviour of the HIF-2 target gene Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED-2), as well as the HIF-1 target genes carbonic anhydrase IX (CAIX) and phosphoglycerate kinase 1 (PGK-1) (figure 7B and C). Consistent with preserved endogenous HIF-1□ and HIF-2□ protein levels (figure 7D), mRNA levels of HIF target genes displayed comparable hypoxic induction in all tested cell lines. Sequencing of the 3'-HRE PCR amplicons derived from gDNA of the mutated clones revealed the presence of two

differentially targeted alleles in each clone (figure 7E). Clone (1) displayed repair of the DSB via NHEJ by insertion of two nucleotides on each allele, while clone (2) displayed one HDR-mediated repaired allele and one small deletion of the locus (13 nucleotides).

In summary, we could show that mutation of the 3'-HRE is sufficient to abrogate Epo hypoxic induction in Hep3B cells. Our results do not provide definitive conclusions on the contribution of the 5'-HRE in regulating oxygen-dependent Epo expression in Hep3B cells as no clone displaying stable and complete editing of the locus could be obtained so far. However, we were able to mutate this locus in HepG2 cells, an alternative Epo-expressing cell line: evaluation of the effect of the 5'-HRE disruption on endogenous Epo mRNA hypoxic levels is ongoing.

Discussion

After characterizing the newly discovered Epo 5'-HRE *in vitro* (Storti et al., 2014), we aimed to mutate the locus in the context of endogenous genomic DNA in Epo-producing cell lines and evaluate the effect on Epo mRNA induction in hypoxia. We additionally applied the same genome editing approach to the established Epo 3'-HRE, in order to assess the contribution of both loci in regulating oxygen-dependent *EPO* gene transcription. In the present study we implemented a novel technique, the CRISPR/Cas technology, which exploits an RNA sequence complementary to the locus of interest to guide the DNA-cleaving enzyme (Cas9) at the desired position. DSB will result in NHEJ or HDR, if a homologous template is provided, and thus in mutation of the gDNA (see introduction). In our hands, NHEJ was more frequent compared to HDR for both tested loci, consistent with observations by others (Saleh-Gohari and Helleday, 2004). Alternative approaches to improve the efficiency of HDR could be to increase the amount of donor template (ssDNA oligonucleotide in our case) or to switch to a dsDNA donor template, i.e. a plasmid containing the homologous region of interest. In the latter case, longer homology arm are required compared to ssDNA (Findlay et al., 2014): increased size of double strand homology arms positively correlated with HDR efficiency *in vitro* (Li et al., 2014).

Genome editing of the Epo 3'-HRE in Hep3B cells was successful and initial screenings resulted in two independent colonies carrying mutated alleles for this locus. *In vivo* experiments previously showed that the 3' enhancer is necessary and sufficient to drive hepatic Epo expression: transgenic mice with mutated HBS within the 3'-HRE displayed absent Epo expression in hepatocytes after embryonic day 14.5 and during adulthood under anaemic conditions. Nevertheless, the enhancer is dispensable during early embryonic stages and renal

expression in adult mice: Epo levels in the kidney were in fact comparable to wt after birth, suggesting that distinct hypoxia-regulated elements are responsible for *EPO* transcription in liver and kidney (Suzuki et al., 2011). In the two mutated clones hypoxic Epo mRNA induction was completely abrogated, despite normal functionality of the HIF pathway, supporting the importance of the 3'-HRE in our liver-derived cell model (figure 7).

Unfortunately, we experienced difficulties in targeting the Epo 5'-HRE in Hep3B cells. Editing efficiency seemed to be lower compared to the 3', as many more colonies had to be screened in order to find at least partially positive clones. The presence of a higher number of alleles could explain why we were not able to easily get a 100% 5'-HRE mutated clone. We indeed do not exactly know how many alleles for the *EPO* locus are present in the genome of Hep3B and the number is likely to be higher than 2, given the tumoral origin of these cells (Strachan and Read, 1999; Alberts et al., 2002; Kim et al., 2013). A few reports in literature studied the karyotype of Hep3B, defined as hypotriploid, without highlighting specific abnormalities on the long arm of chromosome 7 where *EPO* is located (Gray et al., 2000; Wong et al., 2000). The genome of cancer cell lines is however unstable and it is possible that additional modification of the karyotype occurred in culture, despite the fact that at least one report showed stability of Hep3B karyotype after repeated passages in culture (Gray et al., 2000). Our preliminary data on karyotyping of a non Epo-expressing batch of Hep3B revealed an extremely complex copy number situation, with a modal chromosome number of 122 and the presence of 4 to 7 copies of chromosome 7 per analysed metaphase (personal communication from Adriano Guetg, University Hospital Zürich, Zürich, Switzerland). The full procedure of transfection and puromycin-dependent selection, followed by sub-cloning and analysis of genome editing efficiency and Epo levels can take for Hep3B up to three months, during which cells are kept in culture and subjected to relatively high stress. Therefore, it is not easy to prevent aging of the cells and subsequent genome instability while performing this technique. An alternative explanation for poor yield of 5'-HRE targeting could be the cell model used for these studies. As a matter of fact, according to our hypothesis the 5'-HRE represents the hypoxia-inducible contribution to renal Epo expression. It must not be forgotten that the cell model we used is derived from human hepatoma and the KIE could be kept in a "closed" chromatin conformation, limiting the access of the CRISPR/Cas machinery to the DNA. Another epigenetic modification that was shown to inhibit modification of gDNA is CpG methylation (Wu et al., 2014), which can also be found within HBSs (see introduction, (Wenger et al., 1998)).

Since our main goal is to assess the potential role of Epo 5'-HRE, rather than the established 3'-HRE, an alternative approach is needed in order to get stably targeted Hep3B cells. Other gRNA

sequences complementary the 5'-HRE locus are currently being tested for efficient editing. Unfortunately, no alternative PAM sequences can be used to have DNA cleavage directly into the HBS but generating DSB in close proximity of the binding site can lead to deletion of bigger pieces of DNA as well, resulting in disruption of the locus of interest. In order to increase transfection efficiency and to make the entire procedure technically easier, lentiviral transfer vectors are now available containing the sgRNA, the Cas9 gene and the puromycin resistance cassette in one single construct and can be used to generate lentiviral particles for cell transduction. Pre-treatment of cells with hypoxia (0.2% O_2, for 24 h) might help making the locus more accessible for the sgRNA/Cas9 complex, since we have previously observed enrichment of HIF-2α binding under these conditions, possibly indicating "opening" of the chromatin structure (Storti et al., 2014). Repeated transfection cycles with the 5'-HRE targeting vectors would also represent an option, as well as obviously increasing the number of screened colonies. It is fundamental to ascertain that clones are derived from single cells, in order to have a homogenous population of cells with stable genomic background. This can be achieved by increasing the dilution factor and ensuring the cells are well separated when seeded, for instance by passing them through a syringe needle. These aforementioned options are part of ongoing approaches. Furthermore, we transfected with the 5'-HRE targeting vectors another Epo-expressing cell line, HepG2, also derived from hepatocellular carcinoma. Targeting efficiency seems to be higher in these cells (figure 5), possibly due to higher transfectability of HepG2 compared to Hep3B, at least in our hands (data not shown). Indeed, 4 clones displaying disruption of the Epo 5'-HRE could be obtained (figure 6) and are being currently investigated for Epo hypoxic induction. Targeting of the 3'-HRE in the same cells is necessary in order to evaluate the contribution of single hypoxia-driven elements on Epo mRNA levels and is indeed ongoing.

Additional future plans include the generation of double 5'- and 3'-HRE mutant cells, because a putative effect of the 5'-HRE on Epo expression could be visible only in the context of mutated 3'-HRE. Moreover, we are performing chromatin immunoprecipitation as well to evaluate the effect of mutating the 3'-HRE on potential HIF and USF-2 binding (see chapters 3 and 6). An interesting development of this sub-project would be to mutate the CACA repeat, both in the 5'- or 3'-HRE. While it is known that this element is necessary for functionality of both 5'- and 3'-HREs (Semenza and Wang, 1992; Storti et al., 2014), it has never been mutated in the endogenous context. A cell line engineered for the CACA repeat could also be a useful tool to identify the potential protein binding to this region.

In summary, site-directed mutagenesis of the Epo 3’-HRE showed that this element is necessary and sufficient for Epo hypoxic induction in Hep3B cells, while the actual contribution of the 5’-HRE remains elusive. This does not exclude the importance of the 5’-HRE as the hypoxia-regulated element within the KIE because the cell model used is liver-derived and not kidney-derived. Ideally, mutation of the 5’-HRE *in vivo* in renal Epo-producing cells is necessary to draw conclusions on the role of the novel HRE in Epo transcription. Our findings demonstrate that the CRISPR/Cas-mediated approach represents a tool to functionally validate hypoxia response elements in mammalian cell lines and opens new possibilities of exploring regulatory element function, including for instance the cryptic CACA repeat.

Figure 1

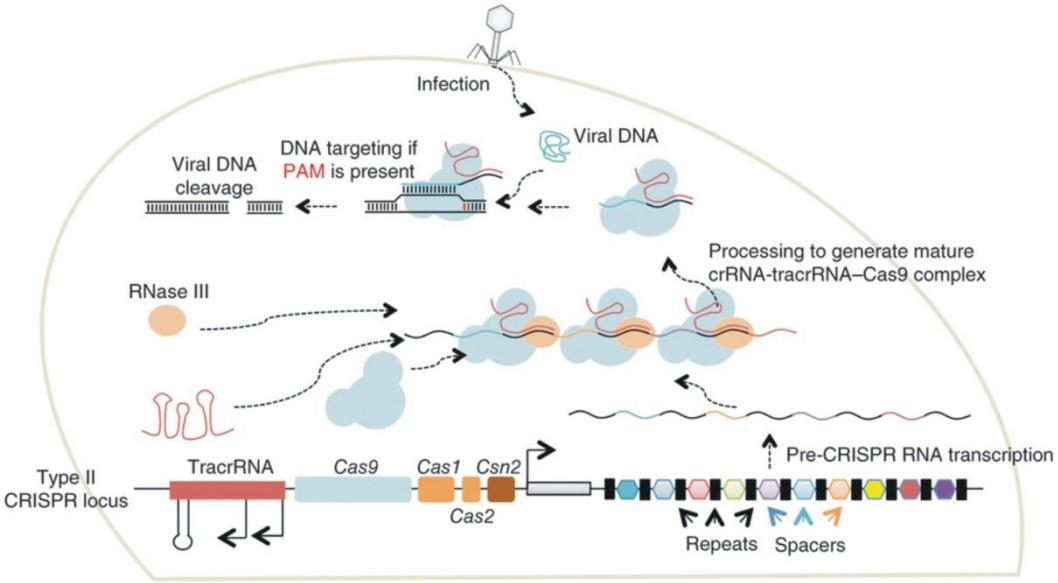

Figure 1 Schematic overview of endogenous type II CRISPR/Cas system: the CRISPR locus is transcribed and processed to produce crRNAs (or gRNAs) that guide effector nucleases to locate and cleave nucleic acids complementary to the spacer. First, tracrRNAs hybridize to repeat regions of the pre-crRNA. Second, endogenous RNase III cleaves the hybridized crRNA-tracrRNA, and a second event removes the 5′ end of the spacer, generating mature crRNAs that remain associated with the tracrRNA and Cas9. The complex cleaves complementary proto-spacers only if a PAM sequence is present (adapted from (Mali et al., 2013)).

Figure 2

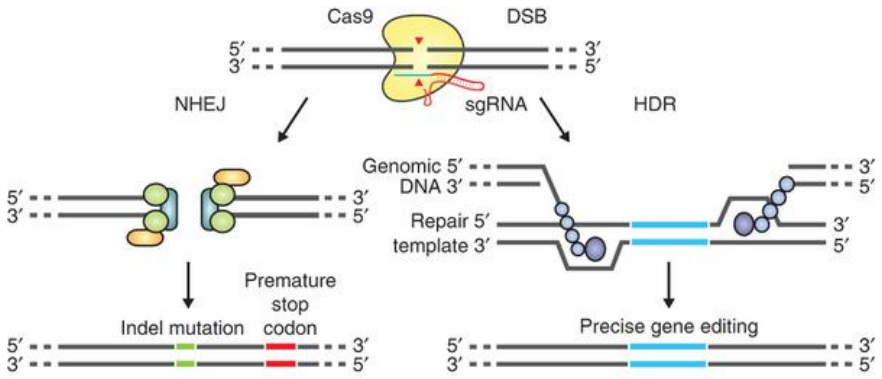

Figure 2 Schematic representation of the two major DNA repair pathways, valid for ZFNs and TALENs as well. DSB: DNA double strand break, NHEJ: non-homologous end joining, HDR: homology-directed repair (Ran et al., 2013).

Figure 3

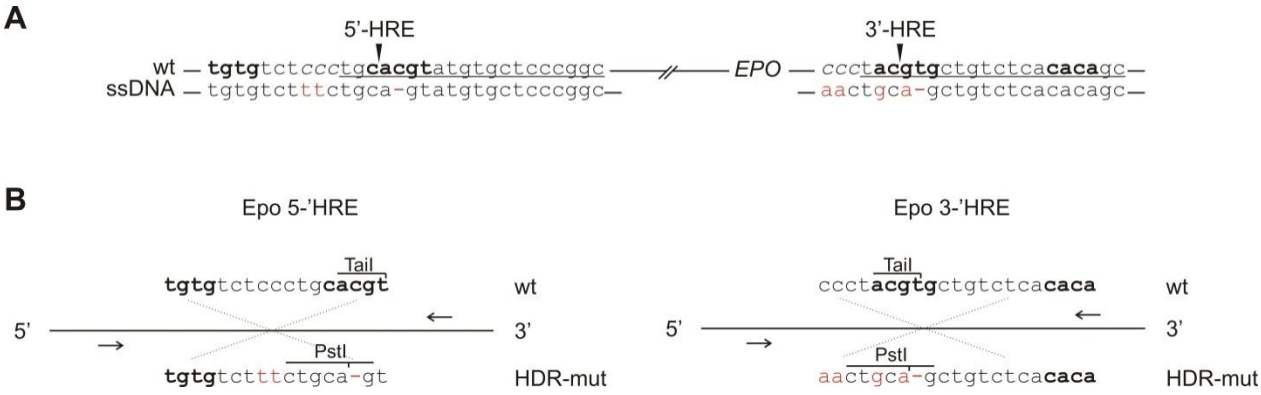

Figure 3 Scheme depicting the strategy used for CRISPR/Cas-mediated targeting of Epo 5'- and 3'-HRE loci. **A**. Wild type (wt) human gDNA sequence from the UCSC Genome Browser database; the HRE (HBS + CACA repeat) is highlighted in bold, the gRNA sequence is underlined and the PAM sequence is marked in italic. The sequences of ssDNA oligos used for HDR are indicated below the wt sequence and the exact position of the predicted Cas9 cleavage site is shown by the arrow. The ssDNA oligos are 100 bp long and contain the desired mutation approximately in the middle. **B**. Restriction analysis of the wt and HDR-mutated Epo 5'- and 3'-HRE loci: the wt sequence contains a TaiI restriction site while the homology recombined one contains a PstI restriction site.

Figure 4

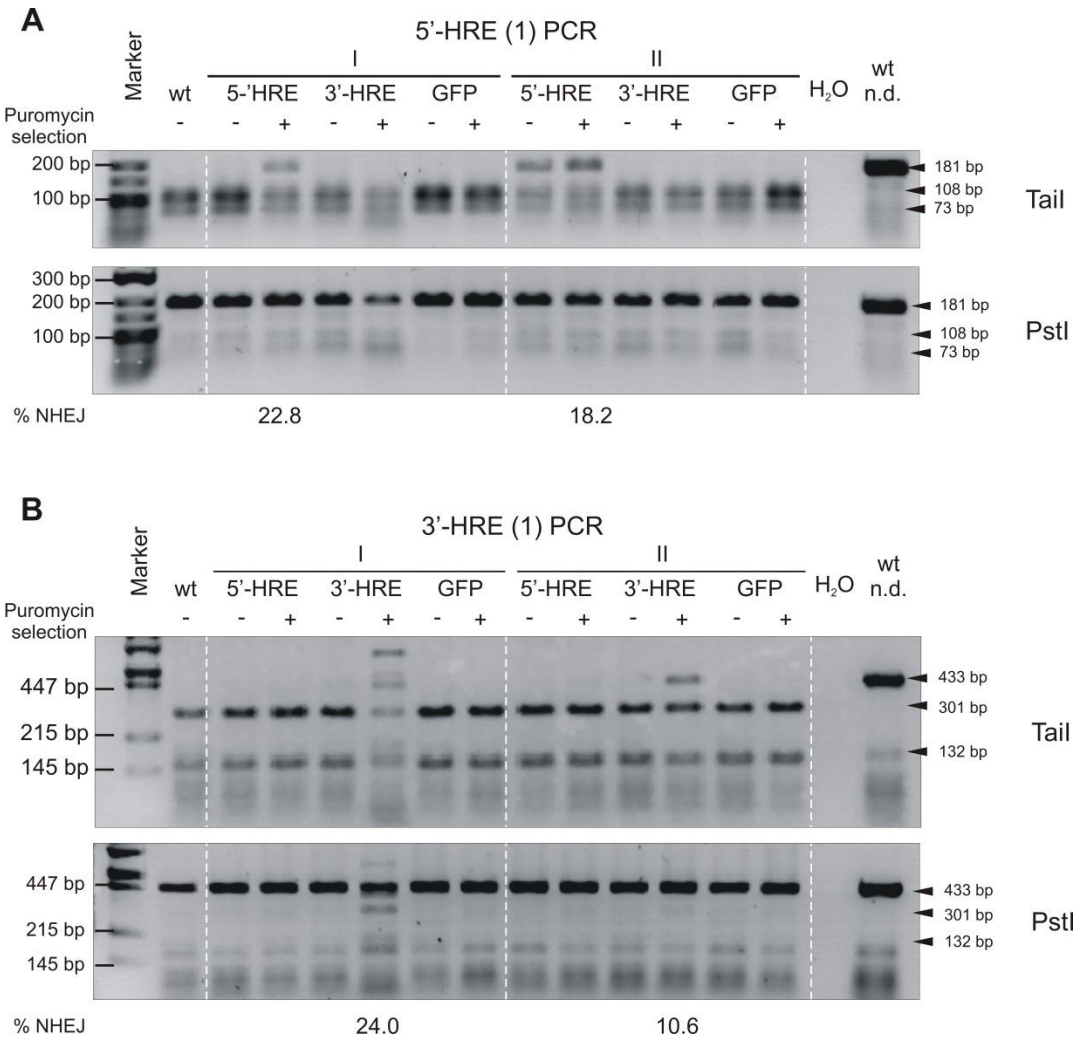

Figure 4 PCR and restriction analysis for Epo 5’- (**A**) and 3’-HRE (**B**) loci on gDNA from wt cells or pools of cells transfected with 5’- or 3’-HRE targeting CRISPR/Cas constructs or a GFP-expressing control (named 5’-HRE, 3’-HRE or GFP, respectively). Cells were harvested before (-) or after (+) 72 h of puromycin selection. Two experiments are shown (I and II). PCR was performed using 5’-HRE primer pair (1) and 3’-HRE primer pair (1), see table 2. Percentage of NHEJ was calculated by quantification of band intensity and the following formula: $a/(a+b+c)*100$, where a is the intensity of the mutated, undigested band normalized on the length in bp, b and c are the intensities of the wt, digested bands normalized on the length in bp. wt n.d. = wt gDNA, not digested.

Figure 5

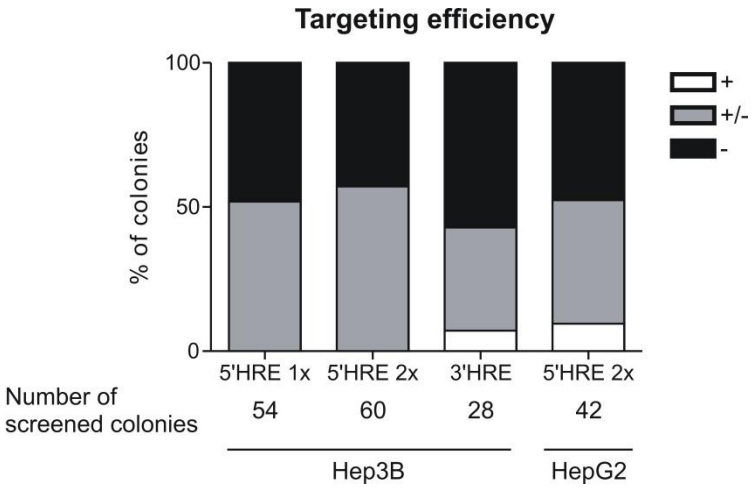

Figure 5 Single cell-derived Hep3B or HepG2 colonies were screened via restriction analysis (figure 3) and the % of wt (-), partially (+/-) or completely (+) mutated colonies was calculated. 5’-HRE 1x/2x = cells transfected once or twice, respectively, with the Epo 5’-HRE CRISPR/Cas targeting vectors. 3’-HRE = cells transfected once with the Epo 3’-HRE CRISPR/Cas targeting vectors. The number of corresponding screened colonies is indicated below.

Figure 6

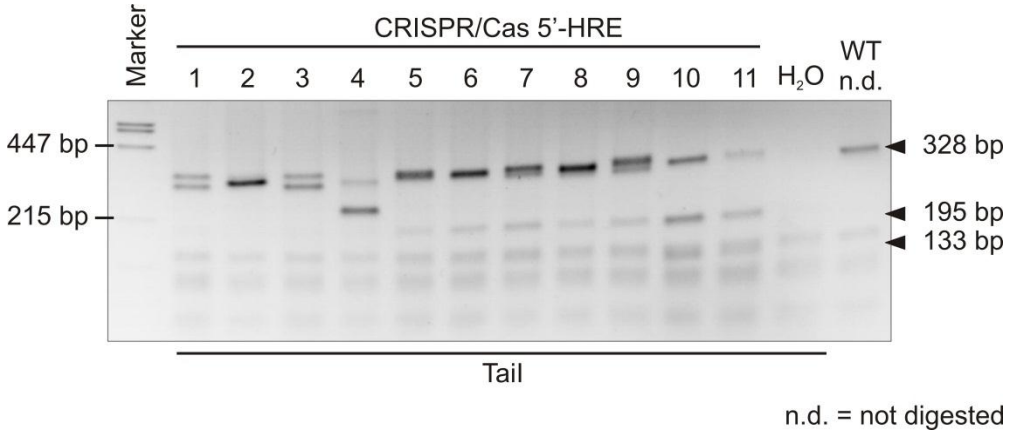

Figure 6 PCR and restriction analysis for Epo 5’-HRE locus on gDNA from wt cells or colonies derived from sub-cloning of HepG2 transfected with the corresponding CRISPR/Cas vectors. PCR was performed using primer pair (2), see table 2.

Figure 7

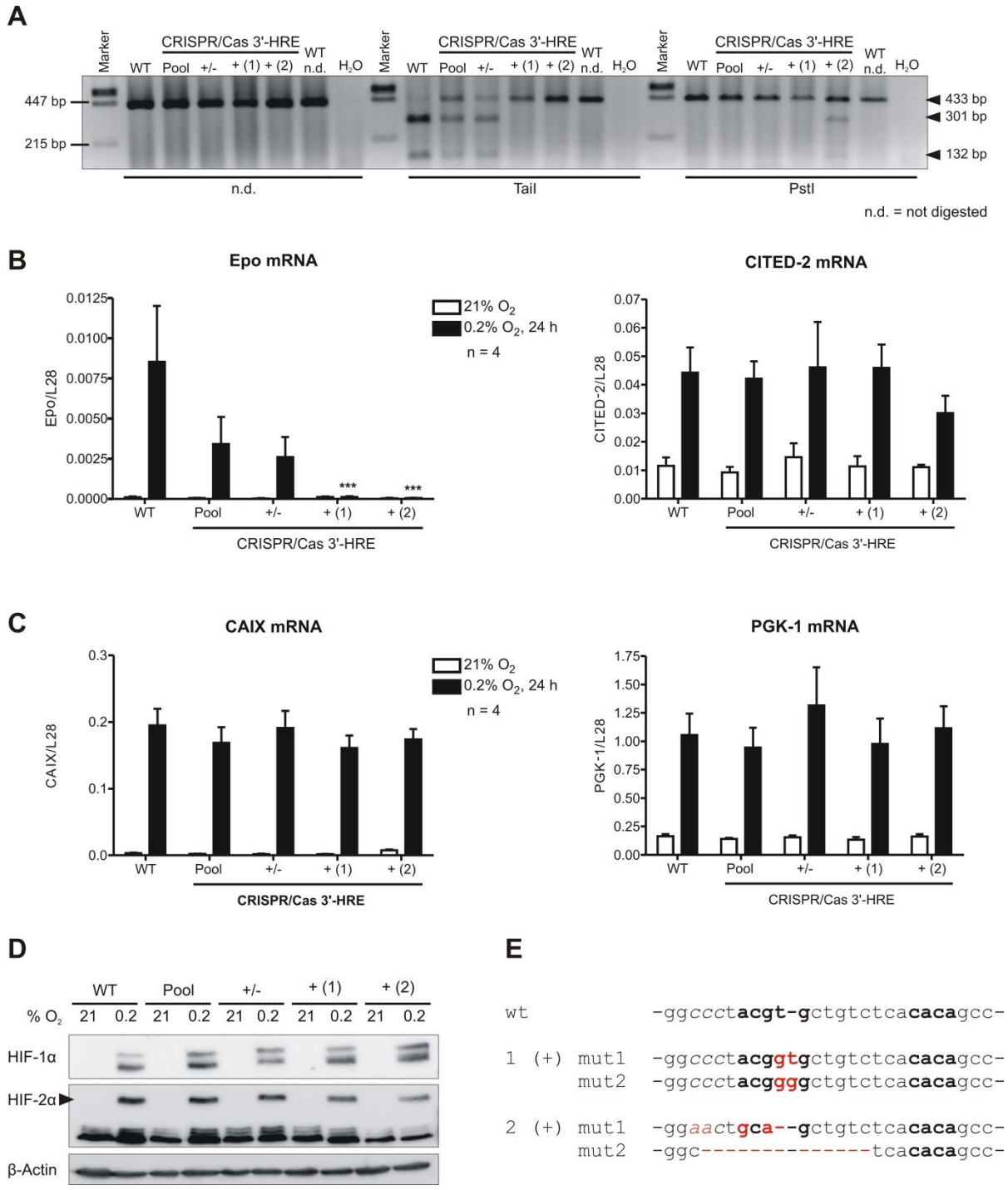

Figure 7 A. PCR and restriction analysis for Epo 3'-HRE locus on gDNA from wt cells or colonies derived from sub-cloning of Hep3B transfected with the corresponding CRISPR/Cas vectors. PCR was performed using primer pair (1), see table 2. The indicated cell lines were exposed to 0.2% O_2 for 24 h and HIF-2 target genes Epo and CITED-2 (**B**), as well as HIF-1

target genes CAIX and PGK-1 (**C**) mRNA levels were measured by RT-qPCR. HIF levels were additionally evaluated by immunoblot analysis (**D**). **E.** Sequence of Epo 3’-HRE in gDNA from wt Hep3B and the two positive (mutated) clones. Pool = mixed population of cells transfected with CRISPR/Cas vectors; +/- and + (1 and 2) = partially and totally mutated single cell-derived colonies.

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9. Conclusions and future perspectives

Oxygen is a fundamental molecule for the life of all aerobic organisms and oxygen sensing through the prolyl-4-hydroxylase domain/hypoxia-inducible factor (PHD/HIF) pathway is necessary in order to maintain tissue oxygen partial pressure (pO_2) at values that are compatible with life (Alberts et al., 2002; Semenza, 2014). One of the most well-known oxygen-dependent genes is erythropoietin (*EPO*), the main regulator of red blood cell homeostasis and thus of O_2 uptake, transport and availability in higher vertebrates (Wenger and Kurtz, 2011). Despite the history of Epo discovery and characterization starts at the end of the 19th century (Viault, 1890), the precise molecular mechanism of transcriptional regulation of the *EPO* gene in the main producing organ, the kidney, remains elusive. The aim of the thesis was to investigate modulation of oxygen-regulated Epo expression by employing the available *in vitro* cell models. In particular, we investigated Epo regulation on three levels:

I. By functionally characterizing a novel hypoxia-response element (HRE) located upstream of the *EPO* gene, within the so-called kidney-inducible element (KIE), and by evaluating the role of a variety of candidate factors on oxygen-dependent Epo regulation
II. By studying the effect of newly identified mutations in the Egl9 homolog 1 (*EGLN1*) gene, encoding for PHD2, associated with secondary congenital erythrocytosis
III. By screening a series of humoral factors and a collection of human blood peptides in order to identify novel HIF, and possibly Epo, regulators

Role of a novel distal HRE and evaluation of different candidate factors in Epo hypoxic induction

Comprehensive *in silico* analysis confirmed the presence of a previously uncharacterized HRE upstream of the *EPO* gene (Epo 5'-HRE), inside the distal region conferring kidney-specificity of Epo expression (KIE), as defined by animal experiments (Köchling et al., 1998; Fandrey, 2004). The presence of a CACA repeat close to the HIF binding site (HBS) resembles the structure of the known Epo 3'-HRE, within the established liver-inducible element (Suzuki et al., 2011; Semenza, 2013). Reporter assays in different cell lines showed hypoxia-inducibility of the 5'-HRE and separate mutation of the HBS or CACA repeat completely abolished the effect of hypoxia, indicating functionality of the HRE within the KIE. Moreover, overexpression of the two HIF-α subunits showed HIF-2 responsiveness of the 5'-HRE and chromatin immunoprecipitation (ChIP) experiments revealed enrichment of HIF-2α binding in hypoxic Hep3B cells, consistently with HIF-2 specificity of Epo expression (chapter 3, (Storti et al., 2014)). Taken together, these data indicate that the 5'-HRE could be the putative hypoxia-regulated element necessary for

renal Epo expression. Indeed, when the 3'-HRE was mutated in transgenic animals, hypoxia-inducible expression of the transgene was lost in the liver but not in the kidney (Suzuki et al., 2011), implying the presence of a different HRE inside the *EPO* locus.

In the context of the newly characterized HRE, we evaluated a series of candidate factors, mainly transcription factors, for their role in Epo hypoxic regulation. We chose to knockdown and/or overexpress these genes and combined this approach with the Epo reporter constructs containing the 5'- and/or the 3'-HRE. None of the factors tested resulted in significant contribution to Epo expression, except for the upstream stimulatory factor 2 (USF-2). USF-2 knockdown led to decreased Epo mRNA levels in hypoxic Hep3B cells, consistent with a previous report (Pawlus et al., 2012), but we could detect binding of USF-2 only on the Epo 3'- and not 5'-HRE (chapter 6).

In order to study the contribution of the 5'-HRE to endogenous *EPO* transcription, we exploited the novel cluster regularly interspace short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) technology for genome editing in Epo-expressing Hep3B. Alongside the 5'-HRE, we mutated the 3'-HRE too, both as a control and to dissect the role of the single HREs in Epo hypoxic induction. In our hands, the CRISPR/Cas technique was very efficient in Hep3B cells for the 3'-HRE locus and we could obtain two clones carrying stably HBS-mutated alleles. Exposure of the 3'-HRE targeted cells to hypoxia revealed complete abrogation of Epo mRNA induction, despite preserved functionality of the HIF pathway (see chapter 8), suggesting that the downstream enhancer is necessary and sufficient for Epo expression in Hep3B cells. Targeting of the 5'-HRE in Hep3B cells was more difficult to achieve and up to now we could not obtain a 100% mutated clone. Many reasons can underlie the difficulties encountered with this locus, in particular polymorphisms in the genomic DNA sequence, chromatin accessibility and epigenetic modifications (Wu et al., 2014). As mentioned in chapter 8, different guiding RNA (gRNA) sequences are currently being tested for higher editing efficiency of the 5'-HRE locus. Moreover, this locus could be kept in a closer chromatin status compared to the 3'-HRE in liver-derived cell lines. ChIP experiments showed indeed binding of both HIF-1α and -2α to the 3'-HRE, while only HIF-2α binding was enriched in our hands on the 5'-HRE (chapter 3): this could indicate more stringent HIF isoform specificity of the upstream element or closer chromatin structure compared to the downstream one. However, DNaseI hypersensitivity assay and methylation analysis of the HBS are needed in order to conclude that chromatin accessibility is the reason for less efficient targeting of Epo 5'-HRE. Also, the presence of more than two alleles containing the *EPO* locus could be a reason for decreased targeting efficiency and the remaining wt allele can be used for homology-directed repair of the mutated ones, resulting in loss of the desired targeting (Wu et

al., 2013). In addition, Hep3B cells do not form easily single-cell derived colonies, a condition that is necessary in order to isolate clonal population of cells carrying the same mutation. Generation of mutated Epo 5'-HRE clones is thus still ongoing and we cannot draw conclusions at the moment about the contribution of the 5'-HRE to endogenous *EPO* transcription in Hep3B cells. However, we were able to mutate this element in an alternative Epo-producing cell line, HepG2: characterization of the 5'-HRE targeted clones is at the moment ongoing. In summary, CRISPR/Cas-mediated genome editing is an efficient and relatively easy technique but success in endogenous genomic DNA stable modification depends on the locus of interest, as well as on the chosen cell model. A number of variations and applications of CRISPR/Cas system have been successfully tested in the last few months, confirming the high degree of flexibility of this novel technology and making it appealing for different experimental purposes (Chen et al., 2013; Fennell et al., 2014; Hsu et al., 2014; Li et al., 2014). To the best of our knowledge, this study is the first to implement the CRISPR/Cas methodology to assess the ability of an HRE in oxygen-dependent transcriptional regulation.

Generating Hep3B carrying stable mutations of the 5'- and/or 3'-HREs opens the possibility for many future perspectives, starting from chromatin immunoprecipitation experiments in order to confirm lack of binding of HIF and additional known or candidate transcription factors binding, including hepatic nuclear factor 4 α (HNF-4α), the GATA factors and USF-2 (ongoing). Furthermore, the 3 dimensional chromatin structure of the *EPO* locus can be explored by performing chromosome conformation capture (3C) assays, a relatively new technique that allows visualization of looping between distant DNA segments, usually promoter and enhancer regions (de Wit and de Laat, 2012; Duan et al., 2012). In this context, genome-edited cells could be used to assess the role of HIFs and other transcription factors in looping of the Epo locus, which was already proposed to occur between the minimal promoter and the 3' enhancer in hypoxic Hep3B cells (Huang et al., 1997; Stockmann and Fandrey, 2006). Interestingly, a recent study by our group investigating the molecular mechanism of oxygen-dependent expression of the transmembrane adaptor protein PAG1 (phosphoprotein associated with glycosphingolipid-enriched mircodomains 1) by a 82 kb upstream HRE confirmed HIF-independent pre-formed chromatin looping (Schörg et al., in preparation). Finally, as mentioned in chapter 8 for the CACA repeat, other binding sites/DNA elements can be mutated via CRISPR/Cas in order to further investigate the molecular basis of *EPO* gene regulation.

Novel EGLN1 mutations causing secondary congenital erythrocytosis

Five different mutations in the *EGLN1* gene were identified in patients suffering from secondary erythrocytosis in an attempt to link genotype and phenotype of the subjects (Ladroue et al., 2012). Importantly, three of them have not been described so far (P200Q, D254H and R398X). Functional characterization of the resulting mutant PHD2 proteins in terms of HIF hydroxylation, stabilization and transcriptional activity led to classification of the mutations into 2 groups:

1. Mutations with weak effect on PHD2 protein activity (P200Q and R371H)
2. Mutations with deleterious impact on PHD2 protein activity, often associated with increased risk of developing tumours such as paraganglioma/pheochromocytoma (R398X, H374R and D254H)

The most interesting mutation from a molecular point of view belongs to the first group (P200Q): no strong difference could be detected between wt and mutant PHD2 in terms of effect on the HIF pathway, despite the patient carrying this mutation displayed the highest Epo plasma levels of the cohort (90 mU/ml compared to a normal range of 5-25 mU/ml). We therefore decided to further investigate in this direction but repetition of the HIF transcriptional and stability assay in a more physiological Epo-producing cell model showed no differential behaviour between wt and mutant PHD2. All the reporter assays performed within this sub-project require overexpression of the PHDs, thus leading to a very artificial situation. PHD2 knockdown or CRISPR/Cas-mediated deficiency and reconstitution of the wt or mutated form up to normal expression levels would be the optimal approach to assess the actual role of the P200Q mutation on functionality of the protein. Other possible explanations include an effect of the mutation on interaction of PHD2 with an unknown factor, particularly crucial for Epo expression. Co-immunoprecipitation experiments would be useful to rule out this possibility or to identify novel Epo-regulatory factors. We cannot at the moment exclude the possibility that the P200Q mutation is not causing the disease, as only the *EGLN1* gene from patients' genomic DNA was sequenced in the study. Indeed, future research efforts aim to study the full exome or genome these erythrocytosis patients (Petousi et al., 2014).

Putative Epo-regulating humoral factors

Since data in literature suggest the existence of humoral factors, such as hormones and peptides, regulating Epo expression in the kidney (see introduction), we selected a number of candidates based on the available evidences and tested them in normoxic and hypoxic Epo-expressing Hep3B. Addition of bovine pituitary extract or steroid hormones (aldosterone and

estrogen, see chapter 7) did not lead to measurable effects on Epo mRNA levels. Consequently, we decided to screen *a priori* a collection of peptides derived from human haemofiltrates (HFs) of patients suffering from renal failure (Schulz-Knappe et al., 1997). These patients display dysregulated Epo expression, thus screening a representation of the peptides present in their blood increases the chance to find Epo regulatory peptides. To this aim, we generated stable HIF-reporter Hep3B cells and optimized a protocol for peptide addition and exposure of the cells to hypoxia. The rationale of the screening is to identify fractions containing bioactive peptides, further sub-fractionate them and test them again in the same assay until the amino acid sequence of a single peptide can be determined (Münch et al., 2007; Borst et al., 2013). We performed the screening twice and found fractions reproducibly displaying an “activatory” or “inhibitory” effect in our HIF reporter assay. We are now at the confirmation step, where we evaluate if the effect is reproducible and truly dependent on the peptide component of the fraction. According to the results of the confirmation phase, we will select the most promising fractions for further steps of sub-fractionation. Given that many HIF regulatory peptides are already known (Wenger et al., 2005) whereas no Epo regulatory peptide has been described so far, we generated novel stable Epo-reporter Hep3B cells, based on the Epo 5’ HRE finding in this thesis, by transduction of the lentiviral constructs depicted in chapter 7, figure 12 (starting from the reporter plasmids used in chapter 3). Testing of these cell lines is currently ongoing and, in case of promising results, they will be used further for stimulation with the fractions selected from the complete screening performed in HIF-reporter cells or for new screening rounds.

Another research line we would like to follow is the inhibitory effect of estrogen on HIF-2α mRNA and protein levels, previously observed in breast cancer cell lines (Jerry H. Fuady, unpublished data). The effect seems to be mediated by both estrogen receptor α and β (ERα and β) which are poorly or not expressed by our Epo-producing cell models (see chapter 7): generation of stably ERα/β overexpressing Hep3B cells represents an option to investigate estrogen effect on HIF-2 and Epo levels.

Final remarks

As a general comment, it must not be forgotten that the cell model used for the aforementioned experiments is derived from human hepatoma and probably recapitulates the situation of Epo regulation in the liver. To understand the actual contribution of the Epo 5’-HRE in regulating oxygen-dependento Epo expression and answer many other questions regarding renal Epo regulation, a kidney-derived *in vitro* model is required. Numerous previous attempts to generate

renal Epo-producing cells (REPCs) growing in cell culture conditions failed and our group is currently working on a mouse model carrying the tamoxifen-inducible Cre recombinase gene under the control of a 220 kb *Epo* gene locus (*Epo*-Cre). This tool will allow specific targeting of REPCs *in vivo* and can be used for isolation of REPCs in culture. Crossing between *Epo*-Cre and a ubiquitous Tomato reporter mouse already showed promising results and fluorescence-activated cell sorting (FACS) could be used to isolate Tomato-positive REPCs. Another idea is to cross the *Epo*-Cre strain with the so-called Terminator mouse, an animal model in which the murine Rosa26 locus was engineered by introducing the diphtheria toxin receptor flanked by loxP sites (Guo et al., 2013). In this way, correctly recombined cells (in this case, REPCs) do not express the receptor and survive in culture with diphtheria toxin-containing medium, an elegant approach to enrich *in vitro* the cell type of interest.

Besides the interest from a physiological point of view, studying the molecular mechanisms regulating Epo production has clinical applications in patients affected by erythrocytosis, anaemia or chronic kidney disease (CKD). Especially for the cases in which Epo levels are sub-optimal, the expensive therapy with recombinant human Epo (rhEpo) could be replaced with oral drugs, combining higher compliance of the patient, reduced costs and side effects (Haase, 2013). As mentioned in the introduction, a number of such compounds, mainly PHD inhibitors, are currently under clinical investigation (Wang et al., 2012; Hong et al., 2013; Flamme et al., 2014). In this scenario, unraveling the molecular basis of *EPO* gene regulation and the characteristics of REPCs can help for instance to find more specific ways to stimulate endogenous Epo production in the damaged kidney of CKD patients.

In summary, the results of this thesis contribute to the understanding of *EPO* gene regulation by characterizing a novel hypoxia-regulated DNA element, by functionally linking different mutations in the gene encoding for PHD2 to secondary congenital erythrocytosis and by evaluating a series of humoral factors for their effect on Epo expression. Whereas the discovery and characterization of a distal 5'-HRE likely representing the long sought-for kidney inducible element regulating Epo expression forms a major achievement in this work, many questions still remain to be answered and the approaches described above will help in the near future to shed light on the complex regulation of the most hypoxia-sensitive gene, erythropoietin.

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10. Contributions to the thesis

Manuscript I: A novel distal upstream hypoxia response element regulating oxygen-dependent erythropoietin gene expression

Federica Storti, Sara Santambrogio, Lisa Crowther, Teresa Otto, Irene Abreu-Rodríguez, Muriel Kaufmann, Cheng-Jun Hu, Christof Dame, Joachim Fandrey, Roland H. Wenger and David Hoogewijs

All figures except figure 2G.

Manuscript II: Distinct deregulation of the hypoxia inducible factor by PHD2 mutants identified in germline DNA of patients with polycythemia

Charline Ladroue, David Hoogewijs, Sophie Gad, Romain Carcenac, **Federica Storti**, Michel Barrois, Anne-Paule Gimenez-Roqueplo, Michel Leporrier, Nicole Casadevall, Olivier Hermine, Jean-Jacques Kiladjian, André Baruchel, Fadi Fakhoury, Brigitte Bressac-de Paillerets, Jean Feunteun, Nathalie Mazure, Jacques Pouysségur, Roland H. Wenger, Stéphane Richard and Betty Gardie

Figure 2B.

Unpublished results: Epo-producing cellular models

Federica Storti, Sara Santambrogio, Irene Abreu-Rodríguez, Joachim Fandrey, Roland H. Wenger and David Hoogewijs

All figures.

Unpublished results: candidate factors involved in Epo regulation

Federica Storti, Lisa Crowther, Alexandra Schörg, Irene Abreu-Rodríguez, Joachim Fandrey, Cheng-Jun Hu, Christof Dame, Roland H. Wenger and David Hoogewijs

All figures.

Unpublished results: Epo regulatory peptides

Federica Storti, Irene Abreu-Rodríguez, Jerry H. Fuady, Ian Frew, Wolf-Georg Forssmann, Roland H. Wenger and David Hoogewijs

All figures.

Unpublished results: CRISPR/Cas-mediated genome editing to dissect the role of endogenous distal and proximal hypoxia response elements in regulating oxygen-dependent Epo expression

Federica Storti, Lisa Crowther, Patrick Spielmann, Roland H. Wenger and David Hoogewijs

Figures 3, 4, 5, 6 and 7.

11. Curriculum Vitae

Surname: STORTI

First name: Federica

Date of birth: 30.06.1986

Nationality: Italian

Education:

- High School Diploma at Liceo Scientifico Statale Giovanni Gandini, Lodi, Italy. Year of graduation: 2005. Mark: 100/100.
- Bachelor of Medical and Pharmaceutical Biotechnologies at Universita' Vita-Salute San Raffaele, Milano, Italy. Length of graduate studies: 3 years. Year of graduation: 2008. Mark: 110/110, *cum laude*.
- Master of Molecular, Medical and Cellular Biotechnologies at Universita' Vita-Salute San Raffaele, Milano, Italy. Length of graduate studies: 2 years. Year of graduation: 2010. Mark: 110/110, *cum laude*. Title of diploma thesis: "Role of receptor tyrosine kinase ROR2 in multiple myeloma: implications for cell migration and drug sensitivity". Subject: Molecular Oncology.
- Employed as a PhD student in Prof. Roland Wenger's lab (Institute of Physiology, University of Zürich) since 1st of December, 2010.

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